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## Please, SIPRI, stay on the fence

AS commentators never tire of pointing out, the presence of the word 'peace' in the title of any organisation immediately renders the intentions of that body suspect; such has been the misuse of one of the most beautiful as well as one of the most significant words of the English language. But the same commentators generally make that point in order to draw attention to the one glowing exception—the Stockholm International Peace Research Institute (SIPRI)—an organisation which in nine years has built up for itself a major reputation as a purveyor of accurate unvarnished information on armament and disarmament provided by an international team whose commitment has been to objectivity in assessment rather than to politically motivated public statements.

Scientists have had particular cause to be grateful to SIPRI. Much of the present debate on arms control and disarmament centres on technological feasibility, prospects held by military research and development and methods of verification. In these fields, in which no national military organisation exactly invites the participation of outsiders, SIPRI has fought bravely to keep abreast and to provide hard fact. The SIPRI Yearbook has become necessary reading for all who profess an interest in defence, of whatever political hue.

Since SIPRI's inception, *Nature* has been both an admirer of the institute and a regular user of its data. (This partly stems from the concerns of both the present and the immediate-past editor in nuclear matters. The present editor (declaring an interest) also worked for a time at SIPRI.) We shall, no doubt, continue to depend heavily on SIPRI for many things, but it is necessary to note a gradual shift in the institute's policy which could jeopardise its future effectiveness, at least if it conceives of its future function as similar to the one it has discharged with distinction in the past nine years.

When SIPRI was founded by the Swedish government and guaranteed its independence, there were those who saw it simply as a private research department of Mrs Alva Myrdal, the Swedish delegate to the Eighteen-nation Disarmament Conference. Certainly Mrs Myrdal has used SIPRI's work more than most, but any fears that the institute would become subservient to a particular philosophy were soon allayed. As well as eschewing external alignments, SIPRI has had to watch its internal scene rather carefully. Of necessity staff who are prepared to uproot their lives elsewhere in order to spend two or three years in Stockholm are going to be of more than average commitment. The institute has never made any secret of this—indeed in the first yearbook the then director, Robert Neild, acknowledged that his staff were of one mind that the arms race was dangerous and that efforts to slow it down had been incommensurate with the danger: SIPRI's skill has been in converting this conviction into objective analysis.

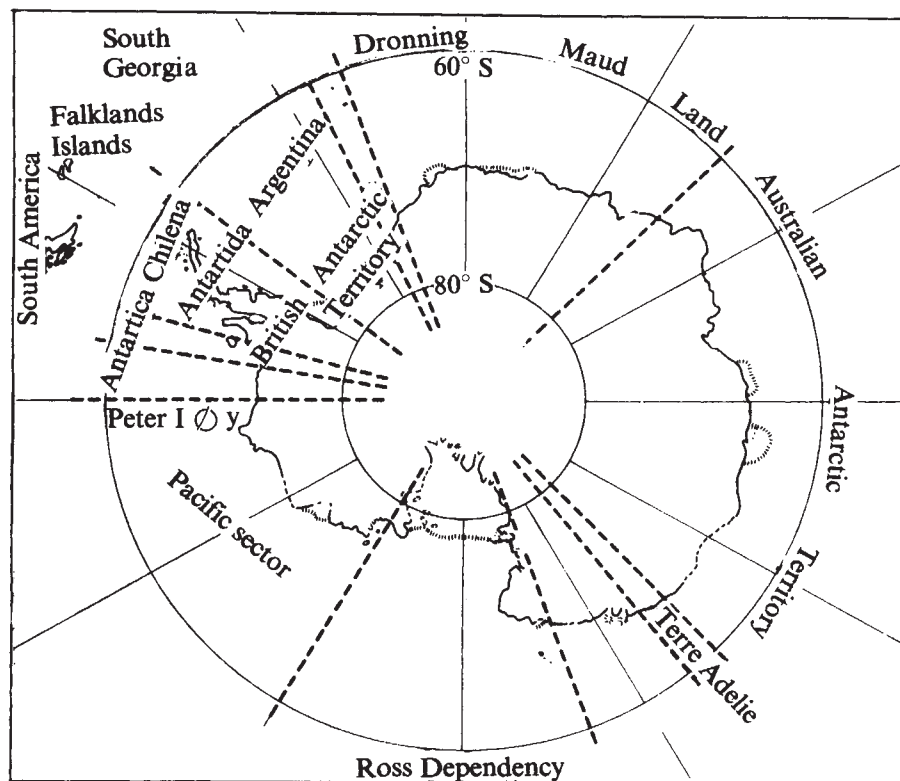
In the past few years there have been signs of growing impatience—not with objective analysis but with political systems which stoke up the arms race and treat arms control with such little respect. Until recently this impatience has hardly manifested itself in very extreme forms; the pages of SIPRI reports don't exactly drip with blood. There has just been the odd paragraph or two in a preface or a summary indicating despair, particularly with the pursuits of partial arms control measures to the exclusion of more general disarmament. Small beer, maybe, compared with the sort of editorialising that everyone else does—and why shouldn't they use their own results more positively? And yet the surprise one feels at encountering even such inoffensive (though controversial) comment is akin to the shock that would be created if the Registrar-General were to preface his annual statistics by a comment that he was getting tired of recording increases in population; people must cut down on copulation.

The most recent publications from Stockholm suggest that the trend towards a more committed viewpoint continues. Two booklets by Frank Barnaby, the present director, (*Preventing nuclear-weapon proliferation* and *Nuclear disarmament or nuclear war?*) are fairly obviously aimed at a new and much wider audience. The emotional temperature has gone up a fair bit, to the extent of a few half-page pictures, not all strictly relevant (such as a napalm victim) and some ringing phrases about nuclear strategy being "inhumane, immoral even genocidal" and so on.

Well, who are we to snipe about emotional writing and irrelevant pictures? The point is simply this. If SIPRI is to become more polemical it will undoubtedly gain in popular acclaim and will always find a ready audience and willing supporters. But it will forfeit its status as an organisation that can work within the system and command respect and help from insiders. There are far too few bodies of this sort in arms control for this to be seen as anything but a matter of great concern.

It is quite likely that much of this change of attitude at SIPRI springs from a feeling of impotence in the face of so much military expenditure. This is to adopt too pessimistic view of the benefits of reliable factual reporting, for which the dividends are always less tangible but ultimately more profound. And SIPRI has had real successes, particularly in its reporting on the world arms trade and its long term study in chemical and biological warfare. These very successes have sprung from the institute's ability to seek opinions and deal directly and honestly with many individuals deeply involved in questions of defence and armament. Come down off the fence on the other side from them and they will neither give advice nor read what is written. □





## Antarctica: the end of an era?

THE problems of mineral exploration in Antarctica will almost certainly be discussed when signatories of the Antarctic Treaty meet in Oslo in June. Largely concerned with the potentially vast reserves of oil and other fossil fuels, the discussions will ensure that old issues of territorial sovereignty will once again be raised. But fears that each of 12 signatory states intend to grant themselves exclusive rights of exploitation may be unnecessarily pessimistic, writes Alan Piper.

There is still a considerable amount of doubt about the full extent and

location of fossil fuel resources in Antarctica, though consideration of the pre-drift, Gondwana continental arrangements suggests that the Ross Sea and Weddel Sea areas may prove productive. Even vague possibilities, however, lend an added significance to the debate about possible mineral exploitation in Antarctica and it is unlikely that the tacitly acknowledged, underlying political rationale behind the maintenance of expensive scientific bases in Antarctica can remain suppressed for very much longer.

At the time that the Antarctic

Treaty (see box) was signed five of the contracting nations — Australia, the UK, France, New Zealand, and Norway — had previously asserted recognised claims to about 80% of the Antarctic landmass and adjoining continental shelf in sectors radiating outwards from the pole. Of the remaining signatories, Argentina and Chile had advanced conflicting claims to most of the British sector, while the governments of the Soviet Union and the United States had refused at any time to recognise any territorial claims on the continent and have asserted none themselves; Japan had renounced her former territorial claims under the terms of the 1951 peace treaty.

The treaty is justifiably held in high regard as a considerable political achievement. It not only froze the legal *status quo* regarding claims to territorial sovereignty but, significantly, was the first multinational treaty of substance to involve the Soviet Union. The demilitarisation of the Antarctic continent and the provision for scientific cooperation has provided a continuing zone of contact between the super powers.

The treaty has worked well in its assurance of exclusively peaceful operations. And though each of the signatories can inspect "the full scope of another nation's scientific and logistics operations", a right regularly exercised by several countries, no evidence has yet been produced to suggest that the spirit of the treaty has been contravened by any party.

Regular consultative meetings of signatories are called for by the treaty, and at the last meeting, held in Wellington in 1972, the representatives recommended to their governments that "the subject 'Antarctic Resources Effects of Mineral Exploration' be carefully studied and included on the Agenda of the Eighth Consultative Meeting". The issue will certainly figure prominently in the Oslo discussions.

To date, there have been no signs that the UK has finalised a policy on the issue of economic exploitation, but some decisions will have to be reached soon. The obvious need is for a regime within which mineral exploitation would be available to any state operating under licence, whether or not a party to the present treaty. That approach to the problem, however, would presuppose that the over-riding issue of territorial jurisdiction had been settled. As long as uncertainties over sovereignty continue, it is doubtful whether signatories would agree to recognise the legal rights of any outsider to operate within a claimed sector.

It is to be hoped, at the least, that a system of licensing under the control of the present signatories will be

The Antarctic Treaty, applying to the entire area of the globe south of 60°S, was signed in Washington DC on December 1, 1959 by Argentina, Australia, Belgium, Chile, France, Japan, New Zealand, Norway, South Africa, the Soviet Union, and the United States. After ratification by all 12 signatory states the treaty entered into force on June 23, 1961, since when it has been acceded to by Czechoslovakia, Denmark, the Netherlands, Poland, and Rumania.

Recognising that "it is in the interests of all mankind that Antarctica shall continue for ever to be used exclusively for peaceful purposes and shall not become the scene or object of international discord" the treaty

- suspends previously existing disputes over territorial sovereignty in Antarctica

- prohibits the establishment of military bases, the explosion of nuclear devices and the disposal of radioactive waste within the treaty area

- invests contracting parties with the right to inspect the full scope of another nation's scientific and logistic operations to encourage open scientific cooperation in Antarctica

- calls for regular consultative meetings between the signatory states for the purpose of "exchanging information, consulting together on matters of common interest pertaining to the Antarctic, and formulating and considering ... measures in furtherance of the principles and objectives of the treaty".

In 1991, after the treaty has been in force for 30 years the contracting parties will be required to review its operation.



adopted. Some of the signatories will arrive in Oslo with no definitive policy in mind. That is almost certainly true in the case of the United States. The likelihood of unilateral action, which has always remained a possibility with that country, cannot as yet be ruled out but the Americans will probably adopt a wait-and-see attitude: "On paper, the United States has a policy; in reality, it does not", says a recent article in *Science*. Whatever policies or

non-policies the signatories take to Oslo, however, there is little hope that they will be published in advance. When preparatory discussions, called especially to consider the problem of mineral exploration, ended in Oslo recently no formal statement was issued.

It would be unrealistic to expect that a settlement will be reached during the Oslo talks. (Discussions on the conservation of the Antarctic seals continued for 10 years before the final proposals

were adopted in 1972. Recommendations on measures for the conservation of Antarctic flora and fauna, first proposed in Brussels in 1964, have still not met with final approval.) But most of the parties to the treaty are unwilling to roll an already snowballing problem further into the future, and many will feel that further prevarication on their part will lead outside countries to question the capability of the signatories to handle the matter satisfactorily. □



## Limits to oncology

*Dr Michael Stoker, Director of the Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, thinks that a levy on money for cancer research should be used to ensure that sufficient 'strategic research', as opposed to 'tactical research', is carried out. (From a lecture given at the dedication of the Seeley G. Mudd Building, M.I.T., on March 6.)*

**E**VEN in these hard times, the support available for cancer research is relatively large by comparison with other biomedical fields, especially in the USA. But, in spite of clear enough aims in terms of alleviation of suffering caused by cancer, the course to be followed is uncharted and the weighting to be put on the various alternatives is largely guesswork. Indeed, the modest progress in prevention and cure of cancer has so far been based almost entirely on empirical judgements and serendipity, followed by well organised development, but it has given no guide to formulation of the principles to be followed for any major and general advance. Close monitoring and direction by the sponsors is, therefore, scarcely possible or at any rate useful. This results in a dilemma concerning the limits of cancer research supported by earmarked funds, particularly for those tackling the more

fundamental aspects of cancer biology.

I shall first consider, as background, some pressure which tend to broaden the limits of cancer research. At an extreme would be the socioeconomic viewpoint, which would consider cancer as a minor problem compared with poverty, population control, pollution and so on. This radical view could consider it justified, and not at all dishonest, to divert cancer funds to the greater good, or to choose research topics, which, though related to oncology, also contribute to the solution of greater social ills. But this almost certainly neglects the wishes of the donors. These may be based on emotion and irrational fears about the implacability of cancer, but it is surely the worst and most dangerous sort of scientific conceit to decide that you know better than the customer what he really wants, at any rate in terms of final objectives.

Then there are those who accept the objective of cancer alleviation, but for whom the modest progress so far, and the lack of a rational approach, means that a better understanding of biological systems as a whole is a necessary prerequisite for any real advance in cancer research. Cancer funds at this stage, therefore, should go predominantly to general biology. This viewpoint is no less strong through its reinforcement by the more personal motives and ambitions of scientists. Most of us would like to do something about cancer, but we are also strongly attracted to the great unsolved problems of wider generality, where advances will bring us the acclaim of our peers.

It is against this background, and the opposing forces of accountability, cost benefit and so on, that the question of relevance, and also responsibility to the sponsor or donor, must be considered. If you gave your money to cancer research, how would you like it spent? At one extreme almost any science, or at any rate biological science, would be relevant, whereas, at the other, cancer research funds should be restricted to programmes with at least conceptual links to practical applications in a finite time.

In some fields of research this dilemma can be acute, for example,

those relating to the molecular and supramolecular organisation of eukaryotic cells. Two main classes of research can be identified in relationship to oncology, and it is helpful to distinguish them.

The first would include any research programme which might have a bearing on cancer, but which is just as likely to lead to benefits in other fields of medicine, agriculture and so on. In other words there is no *a priori* reason to suppose that the resulting advances in knowledge will benefit cancer over and above any other human good. Clearly this research includes the general understanding of living systems and is very long term in its objectives. It is still applied research in that the goal is human health rather than satisfaction of curiosity, but it is multi-purpose, with oncology included.

The second class covers research likely to be more relevant to cancer than anything else. Though other benefits may come, the reason for the research is the special likelihood of increasing the understanding and finally the alleviation of cancer, and not of other human ills. It may still be very much concerned with fundamental aspects of living processes, but those thought to be especially important in cancer.

I shall refer to the former approach as strategic research and the latter as tactical research. They can be distinguished by applying the following questions. Is the research programme under consideration more likely to benefit cancer than other ills? If so, it is tactical. Or does the research include cancer among other possible benefits? It would then be strategic. This requires judgement of outcome and not intent.

In the light of these criteria, I shall mention briefly three research fields which are at present believed to be of high priority and which compete for cancer research funds.

The first is the cell surface, which is at present a subject of such intensive study, much of it with cancer funds. But is the elucidation of the mysteries of the cell surface more likely to benefit cancer than for example the vascular diseases or the primary immunological disorders? Of course there are a num-



ber of changes to the cell surface which are considered to be characteristic of cancer cells, or at least of transformed cells; on closer examination, however, many, even of these, have been found to be manifestations of rapid growth and are almost certainly secondary rather than causal phenomena. This has led to an increasing concentration on the role of the surface in normal cell functions, in relation to both growth and differentiation. It does not diminish the possibility that a change in the cell surface is a primary event in a cancer cell, nor should one forget the particular importance of the tumour-specific surface antigens, but the main thrust of cell membrane research seems now to be much more concerned with normal cell biology; although it is of the utmost importance and one of the most fruitful of all present fields I would nevertheless class a good deal of it as strategic research rather than tactical in its relationship to cancer.

Second, tumour virology is clearly concerned more directly with cancer than anything else and is therefore tactical research. This is not only because such viruses are carcinogens but because of their quite exceptional additional values as models of eukaryotic genetic material containing cancer genes. Nonetheless, it may turn out that the ubiquitous endogenous viruses are as relevant to normal development as to cancer. RNA-directed DNA synthesis, which is now used for all sorts of problems, could carry wide implications over and above its special role in certain tumour viruses, and it does not necessarily follow that all reverse transcription is oncology.

Finally, consider differentiation, a diverse and popular field of research which is particularly difficult to assess in relation to cancer. Differentiation involves changes in gene expression and since cancer also is now widely believed to be a manifestation of gene regulation, strong claims are made for cancer funds for support of differentiation, morphogenesis and so on in a variety of prokaryotic as well as eukaryotic systems. Indeed, there is now an additional justification for cancer funds, with the use of the teratoma as a model system. But it does not follow that even a full understanding of gene expression, for example, in embryogenesis, will necessarily lead to a solution of that other change in cell regulation which leads to cancer. In fact, many would hazard a guess that there is a fundamental difference; namely, that cancer involves a change in the genome and differentiation does not.

Most studies on development and differentiation must surely be classed as strategic research, with possible relevance to cancer as well as to many other

practical problems.

Other examples, such as chromosome structure, protein synthesis and so on, would show that there is really a continuous gradient of research in terms of relevance to cancer. Nonetheless, I would maintain that a position on the gradient can usefully be identified at which cancer changes from one of many potential beneficiaries to the primary potential beneficiary. There will naturally be sharp differences of opinion as to where this point lies in relation to specific research programmes and many will disagree with the examples given. But that does not diminish the general value of distinguishing strategic and tactical research when considering allocation of cancer funds.

It would obviously be simple and administratively tidy to restrict cancer funds to tactical research alone, and to leave the support of strategic research to other sources. It would also be an evasion of responsibility and, in my view, a serious mistake. The vast accumulation of knowledge about cancer and cancer cells is matched only by our abysmal lack of understanding, which simply reflects our inability to solve the complexities of eukaryotic cells. So cancer research funds must surely contribute in some way to strategic research dealing with these fundamental issues. But the method of funding should not encourage deceit and need not necessarily be the same as for the more straightforward tactical aspects of oncology.

There have been many studies of the relationship between fundamental research and useful application, mostly in the physical sciences and engineering, and it is generally agreed that a simple causal chain between a curiosity-oriented discovery in a university laboratory and the emergence of a useful product or other advance is, to say the least, extremely rare. The relationship is complex, and there is much to be said for Harry G. Johnson's concept of basic research contributing to a general pool of knowledge in which the applied scientist or developer fishes and takes samples as he needs them (*Minerva*, XI, 17; January 1972). The result is a lack of temporal correlation between the acquisition of the elements of knowledge and their subsequent appearance in useful application.

My plea therefore is that some mechanism should be found so that cancer funds can be used for the maintenance and expansion of this pool of general knowledge. What I am proposing, in effect, is a sort of levy or tax on cancer money to help pay for its dependence on strategic biology. It was Rothschild who included, in a controversial report a few years ago, an enlightened and more widely applicable

proposal along these lines: namely, that most government laboratories, whatever their objectives, be allowed about 10% of their overall budget for allocation to more basic research, not strictly accountable in terms of relevance. The levy from cancer funds might perhaps be higher but we should not forget the successes of the past decade or so, in Hodgkin's disease for example, chorioncarcinoma and some leukaemias, and in at least the avoidance of lung cancer. These advances have not depended, so far as I can see, on any deep understanding of the molecular basis of living processes, and so long as there is a prospect of continued progress the tactical research responsible must surely receive a substantial share of available funds. Nevertheless cancer research has so far had little effect on the outlook for patients suffering from most of the more common cancers, and it is this which calls, in addition, for the long term strategic approach and appropriate investment.

Leaving aside the actual scale, how might a levy system for strategic research work in practice? Within a single centre devoted to cancer research and dependent on grants and contracts it may be decided internally to allocate a certain share of facilities to strategic research of wider relevance. The proportion may vary considerably between centres. At grant level, applications for a predetermined share of cancer funds could be reserved and judged by a special agency, solely on the basis of scientific merit and without concern for the primary relevance to cancer. For government funding it is important that the contribution, however it is organised, should be deliberately set aside from any earmarked cancer budget and for that matter from other earmarked budgets, for cardiovascular disease and arthritis, for example. The levy, even if small, should be quantified and announced, and it should not be regarded as covered by the existing public expenditure on such things as university research.

The decisions about the strategic or tactical aspects of individual research proposals, and their inclusive or exclusive relevance to human cancer, will still have to be made, no doubt by committees of temporarily faceless peers. But I come back in the end to a rather idealistic view that a great deal of responsibility lies at the grass roots, namely with the individual conscience of the scientist who initiates a programme, and the collective conscience of the group who constitute the centre or institute. And for those dependent on cancer funds, the framework of reference for this conscience should be the donor, be it the reluctant taxpayer, the major benefactor or the sad widow who subscribes her pound or dollar. □

# international news

## Mediterranean poison fish forecast

from Alexander Dorozynski

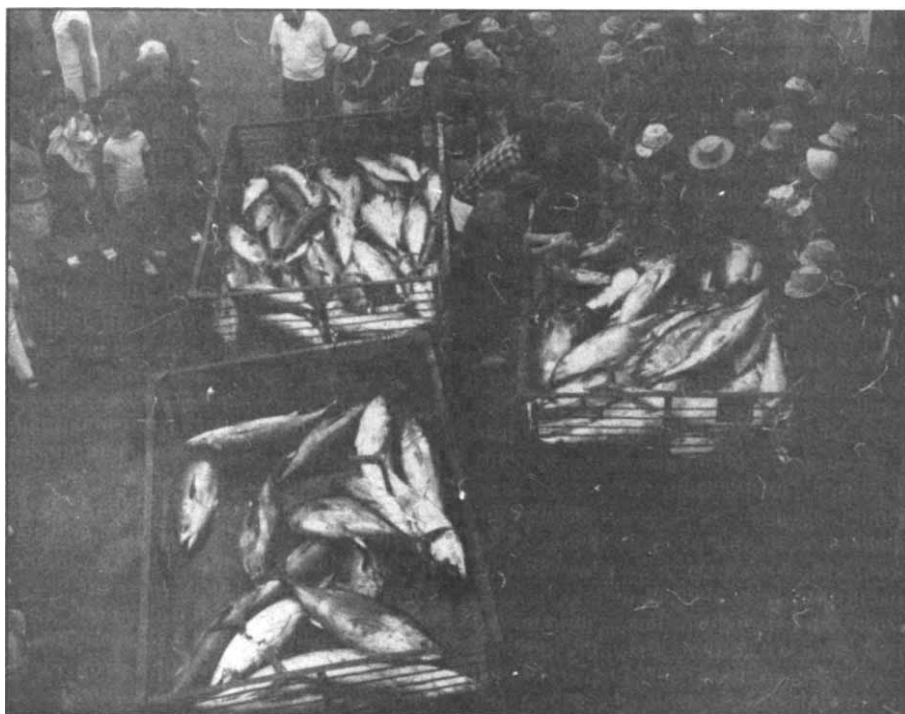
If mercury contamination of Mediterranean fish continues at the present rate, it will take an average fisherman some 375 weeks, or 7 years, to absorb enough mercury for the first symptoms of poisoning to appear, and 1,000 weeks, or about 20 years, for poisoning to become lethal.

This startling forecast is made in a report prepared after the most extensive study of mercury absorption by the biomass yet carried out in the Mediterranean. Maurice Aubert, a medical doctor and director of the Centre d'Etudes et de Recherches de Biologie et d'Océanographie Médicale (CER-BOM) in Nice, has found that average mercury levels in 17 out of 31 species of fish studied exceed the maximum level considered as acceptable in France (0.5 p.p.m.). In nine species (including tuna, swordfish, crab, shrimp and red mullet) the average mercury levels exceed 1 p.p.m.

Dr Aubert points out that previous toxicological and epidemiological studies, notably in Japan and Norway, have shown that a total load of 80 mg of mercury absorbed by the organism of an average adult is lethal, and that the first neurological symptoms appear at about 30 mg. Fishermen along the Mediterranean are known to consume much more fish than other people—a weekly average of 2 kg of fish being a common figure.

Since it has been found that only 4 to 5% of mercury absorbed is fixed in the organism, a weekly absorption of food containing 2 mg of mercury will result in a fixation of 80 µg. At this rate, the lethal dose is reached in about 20 years.

In his report, prepared for the French Institut National de la Santé et de la Recherche Médicale (INSERM), Dr Aubert notes that his estimate is in agreement with pathological studies carried out in Japan among victims of 'Minamata disease'. Among the first victims were children, for whom the lethal dose was 25 µg of mercury. The fish in Minamata Bay contained about 10 p.p.m. of mercury, and the average



*Mediterranean fish market: mercury at dangerous level?*

diet of children included no more than 350 g of fish every week from the age of two until death at the age of five. This means that the total amount of mercury consumed was 3.5 mg a week, or a total of 525 mg during the three years. The accumulated lethal dose of 25 mg corresponds to a fixation rate of 5%.

Statistics concerning adult Japanese fishermen also support his estimates, he writes. From the moment a Minamata fisherman started eating fish containing 10 p.p.m. of mercury, it took about 300 days for the neurological symptoms to appear, and another 500 for the disease to reach the lethal stage. Given an equivalent consumption (and 10 times less contamination of Mediterranean fish), the process would take 10 times longer—somewhat more than seven years for the appearance of first symptoms, and twenty for the fatal outcome.

Dr Aubert, who is senior researcher at INSERM and whose studies are supported by the Ministry of Health, does not want to sound alarmist. A man of the sea and a well-above-average fish eater, he makes it a point to add that there is no reason to panic, to stop eating fish or to deprive fishermen of their livelihood. But there are good reasons for taking stock of the problem and finding ways of reducing mer-

cury seepage into the sea.

Five years ago, Dr Aubert carried out an analysis of hair samples of four regular fish eaters in Nice; two of them were fishermen, one was Dr Aubert himself, and the other was his wife. In one fisherman, he found a total organic mercury concentration of 7.39 p.p.m. In Japan, first symptoms of Minamata disease were found to appear in people having accumulated in hair as little as twice this amount. Another fisherman had 1.58 p.p.m. and Mme Aubert, who is also a medical doctor and works with her husband, 2.88 p.p.m. As to Dr Aubert himself, his hair indicated a non-negligible level of 3.66 p.p.m.

It appears that this is the only study of mercury contamination of man that has been made along the Mediterranean. In Rome, several experts of the UN Food and Agriculture Organisation concerned with the Mediterranean were queried, and none was aware of any other similar study. Yet, the test is neither very difficult to carry out, nor costly.

It is known that the Mediterranean is particularly vulnerable to pollution. Warm and saline, almost tideless, with a limited continental shelf (an extended shelf favours a mixing effect) and communicating with the ocean only by a narrow strait, it is a relatively motionless sea. Most of its shores are densely



populated by people of different nations, some concerned with maintaining their rapid industrial expansion, others with achieving as rapidly as possible a degree of industrialisation equivalent to that of their neighbours.

Anti-pollution measures are not encouraged by energy shortage and economic crisis, and it is not unreasonable to believe that some aspects of pollution are ignored to avoid worrying consumers or restricting producers.

Mercury may be a case in point. Analysis of the mercury content of water is difficult and unreliable, but it is known that even if undetected amounts of this metal are present in water, it can be accumulated along the food chain, reaching particularly large concentrations in long lived and carnivorous fish, such as tuna.

Sources of mercury contamination are numerous and only a thorough investigation can identify them and attribute to each its share. From Barcelona to Genoa, some 50,000 industrial plants, large and small, add metals and chemicals to the sea. It has been estimated that the chemical industry alone is responsible for the rejection of 65 tons of mercury a year. Paper mills, electronic and pharmaceutical plants, and factories making explosives, add their share; so does agriculture, in countries where mercury-based fungicides are not banned. (In France alone, 4.5 tons of mercury are used in agriculture every year.)

Methyl mercury is the compound most readily absorbed by marine organisms. In Minamata Bay, methyl mercury was discharged by an acetaldehyde factory, but Dr Aubert notes that mercury salts can be methylated in the

sea under favourable conditions, particularly in an acid environment. Thus other forms of pollution which contribute to a lowering of the pH of sea water catalyse mercury pollution.

The most typical example of this, he points out, is the result of the dumping at sea of huge amounts of residue by ships of the Montedison factory in Scarlino, on the Italian coast facing the island of Elba. The factory produces titanium dioxide, rejecting among other chemicals and metals, sulphuric acid compounds with a pH of 0.2, causing the lowering of the pH of sea water over large areas. The rejection of these "red muds" (so called because of the colouring action of iron) started "on an experimental basis" in 1972 and went on for two years before the president of Montedison and four administrators were condemned to suspended prison terms.

By then, more than 1 million tons of noxious residues had been dumped north of Cap Corse, disrupting the ecology of an area where some fish were found with almost incredibly high mercury contents (one dolphin had a level of 74 p.p.m. in its liver, 51 p.p.m. in its lungs and 14.6 p.p.m. in its muscles.)

Natural mercury contamination also takes place in the Mediterranean, where deposits are known to exist along the shores. Natural conditions can also contribute to the formation of methyl mercury; sediments, where the pH is lowered by metabolic activity of micro-organisms, can be conducive to the transformation of mercury salts into methyl mercury (this phenomenon was reproduced *in vitro* in CERBOM's aquaria.)

Undoubtedly many other mechanisms are involved, and they are far from being entirely understood; researchers wonder, for example, why is it that in some parts of the sea, where the mercury content is high, the amount of mercury accumulated in the biomass is relatively lower than in other areas, where mercury is hardly detectable.

André Jarrot, Minister of the Quality of Life, points out that the actual mercury levels in fish represent no danger whatever for the average French or European consumer, but announces nevertheless that some measures are being taken.

Already, there is a directive that can ban the setting up in sensitive areas of industrial plants producing chlorine by electrolysis using mercury electrodes. Fungicides containing methyl mercury are no longer authorised and, in the paper industry, the use of fungicides and biocides containing mercury in any form is forbidden. Mr Jarrot points out that a plan is already under way to reduce mercury pollution by 50% in two years and by 90% in five years.

If this is carried out, the French may be as successful in controlling mercury pollution as they seem to have been in controlling bacterial pollution along the beaches, which had reached an unsafe level a few years ago.

Since then, Dr Aubert and other researchers have intensively studied the behaviour of earth bacteria in water; Dr Aubert has identified antibiotic secretions in water and also shown that some marine micro-organisms secrete nucleoproteins that act as "telemediators" (which are inter-specific, but otherwise comparable to pheromones secreted by insects and animals) to trigger or interrupt antibiotic secretion in other micro-organisms. His studies of the antibiotic properties of the sea have led him to develop, in collaboration with Professor N. Desrotte, a mathematical formula to determine the point of discharge at sea of domestic sewage, whether treated or not. The equation is

$$\log C = \log (C_0/h) - (\beta\gamma/1.1)t$$

where  $C_0$  is the bacterial concentration in the sewage before discharge and  $C$  the concentration at time  $t$  (h) after discharge from an outlet at a depth  $h$  (m);  $\beta$  is a coefficient which depends on flow and is unity for a discharge of  $1 \text{ m}^3 \text{ s}^{-1}$ ;  $\gamma$  is a coefficient of the bactericidal action of sea water and is unity for the Mediterranean (comparative measurements in other seas show that  $\gamma$  can vary, so that it is important for it to be included in the mathematical model).

This formula has been used to determine the optimal position of sewage outfalls from large cities, sometimes requiring an outfall several hundred

If contamination by mercury is successfully coped with, another serious problem is nevertheless likely to emerge in the years to come: thermal pollution, notably from nuclear power plants, which are particular wasteful of heat.

So far there is only one nuclear plant on the Mediterranean, at Vandellos, about 30 km south of Tarragona on the Spanish Costa Dorada.

The plant uses the French graphite-gas system, and was built with French technical assistance three years ago. It is cooled with sea water pumped from a depth of 13 m at the rate of  $33,000 \text{ l s}^{-1}$  and rejected directly into the sea. As it enters the plant, the water is chlorinated to prevent shell incrustations in the pipes, and more than 300 tons of chlorine are thus emptied into the sea every year.

The temperature of the water as it leaves the plant is increased by 6 or 7 °C; the temperature of the surface

water is raised by 3 °C over approximately 10 hectares and by 1 °C over several tens of hectares. In the summer, water leaving the plant has a temperature of more than 30 °C—too high for any known Mediterranean life form.

In August 1972, the American ERTS satellite took a photograph showing a large dark area covering several hundred hectares off Vandellos and believed to correspond to an impoverishment of plankton life. But surprisingly enough and in spite of protests of fishermen who have complained that surface fish have disappeared from within a radius of several kilometres from the plant, no serious study has been made of the ecological effects of this thermal and chemical pollution. Such a study could have served as a model for future nuclear plants that are planned along the Spanish coast and in Sète, Martigues and Port-la-Nouvelle on the French side of the Mediterranean.



metres and even several kilometres from the shore so that, whatever the meteorological conditions, the bacterial pollutants do not reach the shore alive.

Thus pollution on the beaches of the Côte d'Azur, which had reached an unpleasantly high level a few years ago, has considerably decreased. Similar improvements are being undertaken in other Mediterranean countries. □

## New deal in French research?

from the staff of La Recherche

FOR the first time since he came to power, M Giscard d'Estaing has invited a select committee on scientific research to the Elysée Palace. The report at the conclusion of this meeting set out in detail the government's scientific policy; in particular, it stressed that "the development and stabilisation of the French research efforts is becoming increasingly important, and France should rate among the leading countries with comparable resources in both the volume and quality of its research . . .". For the first time for many years, the government has thus confirmed its intention to give scientific research some degree of priority.

This political assurance is important insofar as the financing of research has been growing more and more difficult, because recent increases in research budgets have usually been lower than the average rates of growth for general equipment, and barely compensated for inflation. Although the GNP criterion may not necessarily be adequate in determining the research effort of a country, it is nevertheless significant that the portion of the GNP devoted to research in France has been consistently decreasing since 1967, falling from 2.3% to 1.7% in the past seven years. This continuous diminution of the intensity of the research effort, along with serious problems of employment in science, has worried a large section of the scientific community, and the problem was brought to the public eye by two candidates for the presidency of the Republic in May 1974. M Giscard d'Estaing is now attempting to set matters straight in the projected budget, and so to turn away from the policy which he initiated when he was Minister of Finance.

The involvement of the Elysée is without doubt important politically, since it will give a major trump card to those responsible for scientific policy when the next budget discussions are being held—especially to the Minister for Industry and Research, M d'Ornano.

A meeting of ministers at the end of April will decide the main points of

the 1976 budget and it will then become clear whether research is really to be recognised as a priority, and whether the increase in the finance available next year will permit real growth. And now, after the political assurances as to the principle of increased research efforts, and the speculation on the growth of the research budget, it remains to be seen whether French scientific policy will have new life breathed into it.

The first decisions announced after the meeting with the President already provide some useful indicators. On the level of the organisation and construction of research policy, important decisions are of three types. First, provision is made for the formation of executive groups to activate research in certain ministries. Their function would essentially be to coordinate and initiate scientific projects in ministerial departments. On the financial level, the *enveloppe recherche* which includes a large portion of non-scientific research, would be redefined. It will no doubt be extended in the future so as to cover certain other projects, such as the big civil aviation programmes and telecommunications research. This would allow better coordination of scientific policy. Provision is also made for the creation of a basis for intervention in the programme of the Délégation Générale à la Recherche Scientifique et Technique (DGRST). This measure could no doubt be most important for it would, in particular, allow the DGRST to instigate interdisciplinary research projects on various subjects of great general interest.

The second series of measures announced concerns research personnel. It is first stated that science posts will be created "regularly and continuously". Measures will be taken to reabsorb the jobs of researchers without contracts, and to facilitate the mobility of staff, especially concentrating on better coherence of contracts and the devising of appropriate incentives.

The third type of decision aims to involve the scientist more in the responsibilities of scientific policy. It seems that there are divergent views on this. The government has in effect announced, on the one hand, that a committee presided over by M P. Aigrain (and including two academicians) would study reform of the Academy of Sciences and, on the other, that the role and composition of the Consultative Committee would be redefined. This choice can be interpreted as follows. A new, rejuvenated academy, would give the scientific community a forum where ideas could be aired, and a framework within which committees of experts could be formed to report on scientific problems. The Aigrain com-

mittee has already set to work to this end.

The Consultative Committee would be required to translate the wider socio-economic needs of the whole country into the language of the practical objectives and means of scientific policy. On one matter the meeting with the President gave few precise details, that is, on policies relating to industrial research. Certainly, there is mention of the "economic assessment of the research effort" and of the importance of research to aid export growth, but it stays on the level of good intentions. Economic assessment of research was already on the cards in 1966, when the bill was passed creating the Agence Nationale de la Valorisation de la Recherche (ANVAR) on the model of the MRDC; it would be an exaggeration to say that no progress has been made on this problem in the past 10 years, but people have realised that the relation between science and innovation is far from being as simple as they thought.

It is on this problem of policy towards industrial research that the government document probably says least. A special committee under M R. Poignant will be handing in a report very soon on this question to the Minister for Industry and Research. □

## Oceanography in Korea

THE government of South Korea has recently been expanding its interest in science and technology, and nowhere is the interest more visible than in oceanography. Two years ago the Korean Ocean Research and Development Institute (KORDI) was established to coordinate the development of the country's marine resources and collaborate on an international scale. By 1977 the new Science City in Daejeon will be housing KORDI and 16 other institutes in the process of being established; for the present KORDI is based in Seoul.

The growth of the institute depends much on international goodwill. The United Nations Development Programme has agreed to provide \$800,000 over three years, and bilateral assistance is being sought from Japan, European countries and the United States.

A staff of 63 is envisaged by 1977 and recruitment will be a major problem. Since many Koreans with the appropriate experience are living abroad, an extra effort has had to be made to attract them back; salaries are higher than those in comparable government departments, and modern housing is also being made available. □

## Congress to vet NSF grants?

by Colin Norman, Washington

IN a move which is sending shivers of apprehension through the scientific community in the United States, the House of Representatives has decided that Congress should vet every research grant that the National Science Foundation (NSF) wants to award, and that it should have a chance to veto those considered to be a waste of taxpayers' money. A little-noted amendment designed to do just that was attached to an otherwise routine budget bill last week, in spite of an urgent plea by one Congressman that to pass the measure would constitute "an act of public and scientific irresponsibility".

Even if the amendment does not survive passage through the rest of the Congressional mill—it must be approved by the Senate before it becomes law, and its prospects there are far from certain—the fact that the House passed it provides ominous signs of growing political disenchantment with expenditures on some kinds of basic research. Put more bluntly, it is clear that some Congressmen have discovered that many research projects provide plump targets through which they can show the folks back home that they are doing their bit to hold down public expenditure.

The amendment simply states that the NSF must supply Congress each month with details of all the research projects it wants to support, and that Congress should have 30 days in which to exercise a veto over any grant that it considers unworthy. A resolution passed by either the House or the Senate would be sufficient to kill any such project.

Although the amendment does not suggest any criteria by which Congress should judge the scientific worth of

research projects, it asks the NSF to provide "all facts, circumstances and considerations relating to or bearing upon the decision of the National Science Foundation to approve said grants, including to the maximum extent practicable the manner in which the national interest will be fostered by the approval of such grants". Since the NSF awards about 14,000 grants each year, the measure would clearly be an administrative nightmare if it ever reached the law books.

Why has the House suddenly turned sour on the NSF? Part of the reason is the widespread publicity accorded to some broadsides recently delivered by Senator William Proxmire against a handful of the NSF's research programmes, and another factor is that the NSF has been caught up in a bitter controversy over school textbooks. The whole business, in fact, provides an excellent example of the irrational manner in which many policy decisions are taken in the United States.

The NSF's troubles began a few weeks ago when Proxmire, an influential and entertaining man who is closely watched by the press, launched an attack on a few NSF social science programmes by the time-honoured tactic of firing off press releases holding up the research to ridicule. In the space of a couple of weeks, he poured scorn on a study on romantic love, a project entitled "Hitchhiking, a Viable Addition to a Multimodal Transportation System", a study of the "Social Behaviour of Alaskan Brown Bears", and a "Preliminary Investigation of a Special Impact of Television on Blacks".

Even without Proxmire's stamp, the press releases would have been guaranteed newspaper space, for the study on romantic love—which Proxmire termed the "boondoggle of the month"—was so juicy that it was carried by virtually every newspaper in the country, from the *New York Times* downwards. No

matter that the study was designed to investigate why so many marriages end in divorce, which is not an irrelevant factor in family life in the United States, the attacks precipitated a spate of reports of supposedly trivial or useless research projects being supported by government funds. Particularly prominent was a list of projects with funny-sounding names which was broadcast by Paul Harvey, a radio commentator whose utterances are carried by scores of local stations.

Although many of the projects on Harvey's list date back to the early 1960s and have long since been terminated, the upshot of all the publicity was that members of Congress have been deluged with letters from their constituents complaining about wastage of government funds. Since constituents' mail provides an indication of voter sympathies, which few Congressmen can afford to ignore, the NSF suddenly found itself on the receiving end of a good deal of flak.

One example of a trivial research project, which was mentioned during the debate and which has attracted a good deal of publicity, is a \$70,000 study of perspiration in Aborigines. Though that study has attracted much ridicule, it was in fact designed by the Department of Defense in the hope of finding a way to prevent soldiers from becoming dehydrated in tropical climates after many hours without water—a problem which does not seem to be encountered by Aborigines.

Such ill-informed attacks on the NSF's research programmes are by no means new, and they would probably have had little impact this year had it not been for another dispute about the foundation's activities. That dispute concerns a school science course developed in the 1960s by the Educational Development Centre in Cambridge, Massachusetts, with NSF funding. Called "Man: a Course of Study" (MACOS), it is a collection of films,

It is now a year since the European Space Agency (ESA) was to have subsumed the role of ESRO (the European Space Research Organisation) and what was left of that of the European Launcher Development Organisation (ELDO). It was then that ESRO's Director-General Dr Hocker, completed his term of office, making way for an appointment to the new post of Director-General of the ESA. In the event the two got inextricably twisted together and the situation became further confused by the French government's overall review of its science budget and the future of the Ariane launch rocket development which had been accepted as part of the 'European' package.

At last the situation is clarifying. The appointment of a permanent Director-General has been at the root of the whole impasse and it is now clear that the compromise candidate Roy Gibson has been accepted by the two rivals in sponsorship, the French and Germans. They have agreed to drop their respective national candidates and accept the Englishman, who has been doing the job on an acting basis and under considerable difficulties for the past year.

A specially summoned meeting of the space ministers of the 10 member countries, the European Space Conference, is convening in Brussels this week to confirm the appointment. Seven directors to complete the ESA

top management will be announced at the same time. The organisation has been running with three directors short since last year.

It is expected that the same ministerial meeting will decide one way or another on the formula now put forward by the French for ESA participation in the operating costs of the French launch range of Kurou in Guiana from which the Ariane launching rocket is to fly. The switch to the French-designed rocket has also involved a change of launch range. The previous Europa launcher based on the British Blue Streak was launched from Woomera, Australia where at one time the Australians were offering to pay all the operating costs. *Angela Croome*



booklets and exercises designed for use in teaching anthropology to 11-13 year olds. It includes studies of herring gulls, salmon and Netsilik Eskimos—a primitive and near-extinct Eskimo tribe living in North-western Canada. The course is now fully developed, and the NSF has been actively promoting its use in schools.

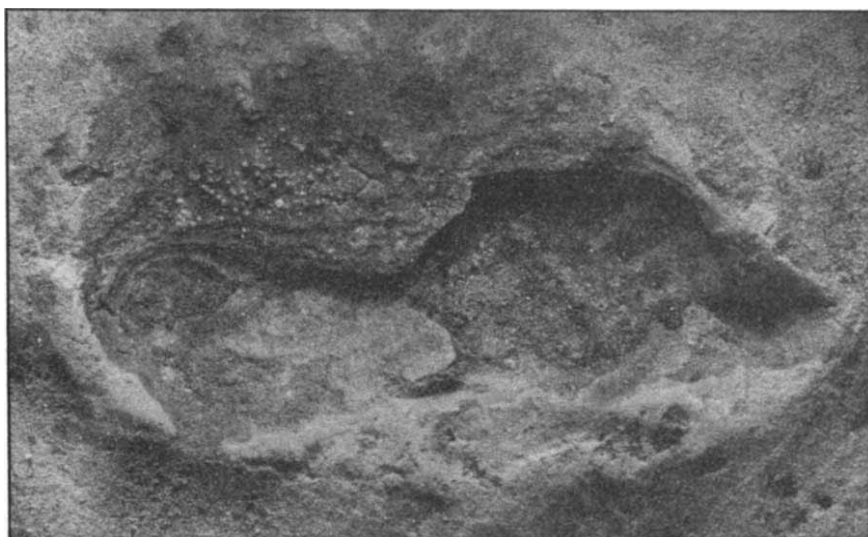
Like a good many other school courses and textbooks, MACOS has, however, generated a good deal of controversy, as a result of which John B. Conlan, a conservative Republican from Arizona, has been trying to persuade Congress to stop the NSF promoting it. Conlan objects particularly to the studies of the Netsilik Eskimos because, he says, "communal living, elimination of the weak and elderly in society, sexual permissiveness and promiscuity, violence and other revolting behaviour" are recurring themes in the studies. About 1,700 schools throughout the United States have, however, decided that MACOS is acceptable and are using the course.

Conlan tried to delete funds from the NSF's budget for promoting MACOS when the matter was being considered by the Science and Technology Committee, but the move failed. The Director of the NSF, Guyford Stever, subsequently announced, however, that he would defer all funding for MACOS pending a complete review of all the NSF's policies for promoting educational courses.

Not satisfied with that, Conlan took his grievances to the floor of the House last week, but failed narrowly—by a vote of 196 to 215—to delete the funds from the NSF's budget. During the course of the debate, Richard Ottinger, a Democrat from New York, suggested that the course simply describes the Netsilik culture in non-offensive terms.

Nevertheless, several members of Congress indicated that they had received many letters from constituents about MACOS, following press accounts of the dispute, and that factor clearly added to the NSF's troubles in the House. Together with the widespread publicity surrounding attacks on supposedly trivial programmes supported by the NSF, the dispute about MACOS indicated to some Congressmen that the NSF should be brought under more intensive scrutiny, and Bauman's amendment seemed to provide a means for doing just that. Consequently, the amendment was approved by 212 votes to 199.

Exactly how Congress should decide which of the NSF's programmes are in the national interest, what criteria should be applied to censorship of educational programmes, and which research could be dropped without hurting the progress of science, is, however, another matter. □



THIS footprint may be 250,000 years old: one of the oldest recorded prints of man. Pressed into volcanic ash, it was uncovered with some others in 1970 during the construction of a dam near Demirköprü in Turkey. It is now on display at the Museum of National history in Stockholm, as part of an exhibition about the origins and future of man.

The Museum acquired the print from Mr Thomas Barton, one of the engineers working on the project in Turkey when the print was uncovered. He did not, however, actually see it extracted as he was working some miles away at the time. He was given it later by men at the discovery site, who told him of its possible interest, and when he returned to Stockholm he brought it with him.

The placard explaining the exhibit states that the Turkish Metal Research Institute in Ankara determined the age of the ash and that the National Laboratory of Forensic Science in Stockholm has examined the print and agrees with the Turkish institute's findings. The forensic laboratory however, denies having tested the ash. According to a spokesman there, two members of staff were only asked to determine whether the imprint was actually of a foot or not. In their opinion it was. Neither has the Natural History Museum itself carried out any tests on the ash, as the equipment at its disposal cannot cope with the supposed youth of the sample. In the opinion of Professor Welin, of the museum's research department, any tests he could do would yield only a very approximate indication of the age of the ash. And there is no equipment elsewhere in Stockholm which could make a more accurate determination.

Apart from the Turkish test, then,

where has the 250,000 year old estimate come from?

The impression of the heel is deeper than that of the instep and the whole print is slightly blurred, suggesting it was made by someone running in the ash. The foot was pointing in the direction of the River Gediz. The theory advanced to fit these facts is that a man was caught in the midst of a volcanic eruption and ran for his life towards the river, imprinting the ash which, because of rain and heat, was baked into that form and shortly afterwards covered with lava. And Mr Barton says that there was volcanic activity in Turkey 250,000 years ago.

If this theory is correct, the print would belong to the Quaternary period. The Museum states that the print predates Cro-Magnon man, who came to Europe at the end of the last ice age. If this is true the runner would have lived during the Pleistocene period—possibly at the same time as Neanderthal man.

As the dating of the print—and even the circumstances of its discovery—have been based largely on hearsay, it would be a good idea to confirm the results of the Turkish test. There are, however, no plans to do this. Only the Turkish Embassy in Stockholm is showing interest on the grounds that Mr Barton did not obtain the necessary official permission to export an antiquity from the country. (Mr Barton insists that he acted within the law in bringing the print back.) During the course of its enquiries into this aspect of the affair the Turkish Foreign Ministry may of course discover whether the print is fact an antiquity or not. But that seems a rather back-handed way of finding out the truth about what could be an important anthropological discovery.

Wendy Barnaby



THE problems of higher and specialist education have always been particularly difficult for Soviet educationalists, who must seek the best means of using potential talents for the good of the state, while not departing from the theoretical tenets of equal opportunity. During the half century of Soviet rule, a number of solutions have been proposed. At one stage the exceptionally gifted were expected to develop their talents in extracurricular time (traces of this remain in the mathematical "olympiads" for schoolchildren), whereas under Khrushchev, the policy was one of what may broadly be called 'sandwich courses for all'—a coordination of education and work which led to considerable interruption of production and the exasperation of supervisors and managers. During the past few years, the Khrushchev policy has been quietly modified, adapted and legislated out of existence, and a return to more conventional policies introduced.

The new approach includes a certain amount of streaming, or rather creaming, of the highly gifted into specialist schools at a fairly early age. (One interesting facet of Soviet educational psychology is that, although it denies the existence of educational subnormality—except in the case of actual brain damage—it does admit the existence of above-average mentalities—although hedging its bets to the extent of claiming that the intelligence of a "lively" average child can be improved by educating him or her together with the highly gifted). Whatever the theoretical justification for creaming, current Soviet policy seems to have as its pragmatic aim the production of the vast work force of the intelligentsia needed for the expansion of the Soviet economy.

A recent *Pravda* article by the Minister of Higher and Specialist Secondary Education, V. Elyutin, outlines the plans for the 1980s and 1990s, and stresses the need for a new strategy in scientific and technological training for the next 10 years. Present plans include means of "making the training of specialists more flexible, and ensuring the organic connection of education and life, science and industrial activity". One defect of the Khrushchev system was that, for logistical reasons, young people were assigned to factory training that was unrelated to their talents or future professions. Another fault was the difficulty secondary school children had relating their school studies to the specialist technique of industry, in which they were supposed to participate. Minister Elyutin's stress on the cooperation of higher educational institutions (from Moscow Uni-

versity downwards) with industry—a policy which is to be "extended further" in the immediate future—indicates that the emphasis will now be on such industrial training at a later age, when it can be more meaningful to the student and less disruptive to the factory. He notes, however, that such cooperation between institutes, or groups of institutes, and industrial enterprises may take "the most diverse" forms. The new plans are still production-orientated ("changes in specialities and specialisations" will be introduced

## Russia today

from Vera Rich, London



"in accordance with the needs of the national economy as they arise"), yet underlying the whole statement one senses a realisation that such a policy can only be fruitful if it is implemented in accordance with the special circumstances of each institute.

● The failure of the latest Soyuz to complete its link-up with the orbiting Salyut space station will not, repeat not, have any effect on the joint Soyuz-Apollo mission planned for July 1975. So we are told quite firmly by both parties to the project. Commander Richard Truly, the back-up capsule commander of the US team, said at a press conference in London, on his way to Moscow for a joint training mission, that the Americans still have every confidence in the joint mission and are satisfied with the safety aspects of the flight. The Russians, for their part, were eager to explain that this Soyuz was not the model which will be used for the joint mission, but an earlier version that had been "less diligently" checked than the system planned for the link-up. International goodwill and cooperation continues unaffected.

Yet in spite of the mutual reassurances, one wonders why the Soviet planners chose to announce the failure

at all. It had not risen above the horizon of foreign tracking stations and need therefore never have been mentioned. Presumably it was the forthcoming joint mission which prompted the Soviet planners to make the announcement—but was their motivation one of cooperation, or fear of further setbacks? In the latter case, an aborted flight with the safe recovery of the cosmonauts might serve as a reassurance: even if the rocket fails, the men are safe. The old rumours of Vladimir Ilyushin, Gagarin's alleged predecessor on the launch pad, have never been entirely squashed in either East or West, and the Soyuz failure does offer a certain "substantiation" in that a crew-carrying rocket can be launched and fail without being tracked by foreign stations. The latest Soyuz venture is, at all events, a success for Russian safety procedures, and this fact may well have motivated the decision to release the news.

● Intimations that increasing pressure was to be exerted on participants in the illicit Sunday seminar for "refusnik" scientists have, unhappily, been justified by recent events. A letter to western "Academies of Science, Scientific Societies and Individual Scientists", signed by 45 participants of the seminar, states that increasing pressure is being exerted on individual participants "in order to interfere with the seminar's work." Methods mentioned include the issue of call-up papers for retraining in the Soviet Army (which could later be interpreted as access to classified information, a *prima facie* reason for refusing a visa), prosecution for "parasitism" (being without employment, although the scientists concerned have been deprived of their jobs as a result of applications for exit visas) and, in the case of Mark Azbel, the physicist, a kind of *de facto* exile. Professor Azbel, formerly Head of the Department of Electron Theory at the Landau Institute of Theoretical Physics of the Soviet Academy of Sciences, had been visiting relatives in Chernovtsy, and was about to return home to Moscow when he was stopped at the railway station and told not to go there for several months "or he would find himself further east" (in Siberia). Azbel is now stranded in Odessa without friends or money. It seems that, since the seminar continues to meet, in various venues, the authorities are now trying to erode it by pressure on the individual participants. They still seem unwilling to take the simple step, suggested by the seminar's founder, Aleksandr Voronel, of granting the whole group exit visas and thus letting them transfer their vexing activities to Jerusalem or Tel-Aviv.

# news and views

## Topography of ribosomal proteins

from a Correspondent

THE bacterial ribosome is the only cellular organelle for which a thorough understanding of both structure and function at the molecular level can be expected in the not too distant future. A central part of the structure-function correlation is obviously elucidation of the spatial arrangement of proteins within the ribosome; current interest in this aspect of ribosomes alone has produced a flood of well over a hundred papers in the last twelve months. Here I shall summarise briefly the present state of this problem of 'protein topography' with reference to the *Escherichia coli* ribosome.

In considering the three-dimensional arrangement of the proteins, a number of points must be borne in mind. First, the ribosomal proteins not only vary considerably in size, but, more important in this context, they may also have very irregular or elongated shapes within the ribosomal particle. Second, the proteins are not all present in equimolar amounts at all stages of the protein synthetic cycle; although this question of which proteins are 'unit' and which are 'fractional' still requires some clarification, it is certain that the ribosome cannot be considered a homogeneous entity. It remains to be seen whether the removal or addition of a fractional protein can seriously distort the arrangement of the other proteins. Third, conformational changes can occur as a result of interaction between the ribosomal sub-particles, or as a result of interaction with the various components of protein synthesis (tRNA, factors, and so on) and again it remains to be seen whether such changes are accompanied by a radical change in the protein arrangement within the particle. Fourth, only a small proportion of ribosomes are active in protein synthesis in *in vitro* systems, which raises the possibility that distortions of the protein arrangement may occur as a result of the isolation procedures used, or of course as a result of the procedures used to make the structural investigations. But since several techniques have been used during the last few years to examine the spatial arrangement of the ribosomal proteins, their success may be

judged by the measure of agreement between their various results.

The use of bifunctional protein cross-linking reagents is the most direct way of determining which pairs of proteins are neighbours within the ribosome. The chief problem here is in identifying the proteins contained in the cross-linked complexes; this identification can be made directly with antibodies specific to the individual proteins (for example Lutter, Bode, Kurland and Stöffler, *Molec. gen. Genet.*, **129**, 167; 1974). Most attention however has been given to the development of cleavable reagents, which enable the proteins in the cross-linked complex to be identified by gel electrophoresis, after chemical cleavage of the cross-link. The most widely used reagents for this purpose have been the *bis*-imido esters, and of these, *bis*-methyl suberimide proved in earlier work to have the most useful properties (Bickle, Hershey and Traut, *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1327; 1972). The reagent attaches to the  $\epsilon$ -amino groups of lysine residues, and the cross-link is cleavable by ammonolysis.

Some authors, however, finding that the cleavage reaction did not proceed very satisfactorily, developed other reagents. The most noteworthy new reagents are methyl 4-mercaptobutyrimide (Traut, Bollen, Sun, Hershey, Sundberg and Pierce, *Biochemistry*, **12**, 3266; 1973), and *bis*-azide compounds derived from tartaric acid (Lutter, Orandel and Fasold, *FEBS Lett.*, **48**, 288; 1974). Both of these react with amino groups, but the former does not of itself introduce a protein-protein cross-link. Instead, the mercaptobutyrimide effectively adds an SH group to the protein, and these SH groups can subsequently be cross-linked by mild oxidation. Cleavage of the isolated protein complexes is accomplished in the mercaptobutyrimide case by reduction, and in the tartryl diazide case by periodate oxidation of the vicinal OH groups. Using this approach, many 30S protein pairs have been cross-linked, as have a smaller number of 50S protein pairs (Clegg and Hayes, *Eur. J. Biochem.*, **42**, 21; 1974). The possibility that some of the reagents

can themselves distort the protein arrangement remains, but the consistency of the results obtained with the different reagents argues against this. Further, in one case, a cross-linked protein pair (S5-S8) was incorporated back into a reconstituted 30S particle, without impairment of protein synthetic activity (Lutter and Kurland, *Nature new Biol.*, **243**, 15; 1973).

If the 30S ribosome is treated with nucleases under suitably mild conditions, it can be broken down into fragments of ribonucleoprotein which can be analysed for their protein and RNA content. Those proteins which are found together in such fragments are presumably neighbours in the intact particle, since it is unlikely that a ribonucleoprotein complex consisting of widely separated groups of proteins joined by an unprotected RNA strand could survive the nuclease treatment. Using this approach, the 30S particle can be readily split into two fragments of unequal size, the one containing eight to ten proteins and the other five or six (for example Morgan and Brimacombe, *Eur. J. Biochem.*, **37**, 472; 1973). Various smaller fragments have been isolated, containing sub-sets of these two groups of proteins, the smallest consisting of only two proteins.

A similar division of the 30S proteins into groups has been made from hydrolysates of a protein-deficient reconstitution intermediate (RI) particle (Zimmermann, Muto and Mackie, *J. molec. Biol.*, **86**, 433; 1974). In this type of approach, the proteins are of course not covalently attached to the RNA, and therefore special care must be taken to ensure that the observed results are genuinely specific. The importance of this point has been underlined by the finding that under conditions which promote 'unfolding' of the ribosomal sub-particles, the proteins lose their specific sites of attachment to the RNA (Newton, Rinke and Brimacombe, *FEBS Lett.*, **51**, 215; 1975). This almost certainly invalidates a number of previous fragmentation studies, particularly in the case of the 50S particle, although more recently some specific 50S fragments have been



described (for example Roth and Nierhaus, *J. molec. Biol.*, in the press). But in contrast to the 30S particle, the 50S cannot be split into two well-defined halves, despite the fact that the 23S RNA is readily cleavable into an 18S and a 13S RNA (for example Allet and Spahr, *Eur. J. Biochem.*, **19**, 250; 1971). The finding by neutron scattering that the centres of mass of protein and RNA are widely separated in the 50S particle as opposed to the 30S could explain this difference (Moore, Engelman and Schoenborn, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 172; 1974).

It has been known for some time that the 30S proteins interact with one another during *in vitro* reconstitution from protein and RNA in a very specific manner. These interactions have been incorporated into an 'assembly map' (see Held, Mizushima and Nomura, *J. biol. Chem.*, **248**, 5720; 1974, for the latest version). Although these interactions need not necessarily be a direct reflection of the protein neighbourhoods within the 30S particle, there has been striking agreement between those proteins which are found together in cross-linked pairs or ribonucleoprotein fragments and those which are related in the assembly map. It seems reasonable therefore to conclude that most if not all of the assembly interactions are indeed direct reflections of the ribosomal topography. The question of whether all the proteins in the complete particle have substantial contact with the RNA has yet to be settled, and it has been suggested that it may be more appropriate to think of the assembly interactions as being between regions of ribonucleoprotein as opposed to simply interactions between proteins (Kurland, *J. supramolec. Struct.*, **2**, 178; 1974).

There is of course as yet no corresponding assembly map of the 50S particle, since a successful total reconstitution of the *E. coli* 50S particle has only recently been achieved (Nierhaus and Dohme, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4713; 1974). Some assembly interactions have however been determined, using protein-deficient core particles as the starting point (Highland and Howard, *J. biol. Chem.*, **250**, 831; 1975).

Differential reactivity to chemical reagents has been used by many workers as a probe of ribosomal topography, the assumption being that those proteins which are most exposed on the ribosome surface will react most strongly with a particular protein reagent. Among the methods used have been digestion with trypsin, or reaction with kethoxal, various aldehydes and N-ethyl maleimide. Interpretation of the results of such experiments is however rather complex, since exposure or lack of exposure of a particular re-

active group is not necessarily a reflection of the protein topography in the wider sense. A reactive group on a protein could be shielded by RNA, or by the tertiary structure of the protein itself. Further, it is not easy to predict how far a chemical reagent can penetrate into the ribosome, and it is therefore not surprising that the degree of agreement between the various results is not very high. A good summary of the recent data can be found in a paper by Benkov and Delihias (*Biochem. biophys. Res. Commun.*, **60**, 901; 1974). At present, data from reactivity towards very large reagents are more easy to interpret, and two methods are noteworthy in this context. The first of these is measurement of the accessibility to protein-specific antibodies (for example Stöffler *et al.*, *Molec. gen. Genet.*, **127**, 89; 1973), and the second is the iodination of ribosomal proteins catalysed by lactoperoxidase, of which a recent example is the work of Litman and Cantor (*Biochemistry*, **13**, 512; 1974). The general conclusion from all these methods is that all 30S proteins have some accessible groups on the ribosome surface, whereas the 50S proteins are not so readily accessible.

Several groups have attempted to coordinate the available data into three-dimensional models of the protein arrangement of the 30S particle. In all these models, the proteins have been represented as spheres, and the authors have been careful to point out that the arrangements are only schematic, and serve mainly as a means of testing the self-consistency of the various data. A technique has, however, recently been developed which casts doubt on the whole validity of even this crude type of model building: the direct visualisation of the proteins by electron microscopy of complexes formed between

ribosomal sub-particles and protein-specific immunoglobulins. This technique has become possible since both sub-particles have a readily recognisable shape in the electron microscope. Thus, if the ribosome is treated with a single protein-specific antibody, a ribosome-antibody-ribosome complex is formed, with the Fab arm of the antibody attached to a point on the ribosome surface. Several proteins have been localised on the surface of both sub-particles by this method (for example Tischendorf, Zeichhardt and Stöffler, *Molec. gen. Genet.*, **134**, 187; 1974), but the most important feature of the results is that while some proteins show a single surface binding site for their cognate antibodies, others seem to have multiple binding sites over a wide area of the surface, for example protein S4 (Lake, Pendergast, Kahan and Nomura, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4688; 1974). This indicates that the conformation of the proteins within the ribosomal particle is variable, and can be highly extended, which, as implied at the beginning of this article, alters the whole conception of the ribosomal topography, and rather changes the interpretation which must be made of many other data (such as the protein cross-linking results). In this context it should, however, be borne in mind that the overall dimensions of the 30S particle as measured by electron microscopy differ significantly from those estimated by low angle X-ray scattering in solution (Hill and Fessenden, *J. molec. Biol.*, **90**, 719; 1974).

In conclusion, the topography problem is still obviously some way from complete solution, but it is encouraging that the techniques in current use are by no means exhausted; further rapid progress can therefore be expected.

## Devonian arthrodires

from B. G. Gardiner

A. RITCHIE's article (see this issue of *Nature*, page 569) on Devonian arthrodires is a painstakingly compiled record of the distribution of the genus *Groenlandaspis*. He has shown that—like some other arthrodires (including *Bothriolepis*, *Holonema*) — *Groenlandaspis* is found in Devonian deposits all over the world and pleasingly has confirmed that the poorly known *Coccosteus disjunctus* from Ireland and Bristol is yet another species of *Groenlandaspis*.

The distribution of *Groenlandaspis* will certainly excite palaeogeographic interest, but it is dangerous to jump to hasty conclusions about continental drift from such evidence

and Ritchie's caution is amply justified. As yet there is no articulated theory to enable us to deal with the zoogeography of Devonian fishes and so the significance of their distribution, which has been a puzzle for many years, remains an enigma. For most groups of fishes this will only be resolved when we have a clear picture of the configuration of the continents in the Devonian. But in the case of arthrodires, an entirely fossil group, an even more critical component is missing. For them we lack an acceptable phylogeny, a vital factor because palaeozoogeography can only make sense as the evolution of organisms in space and time.

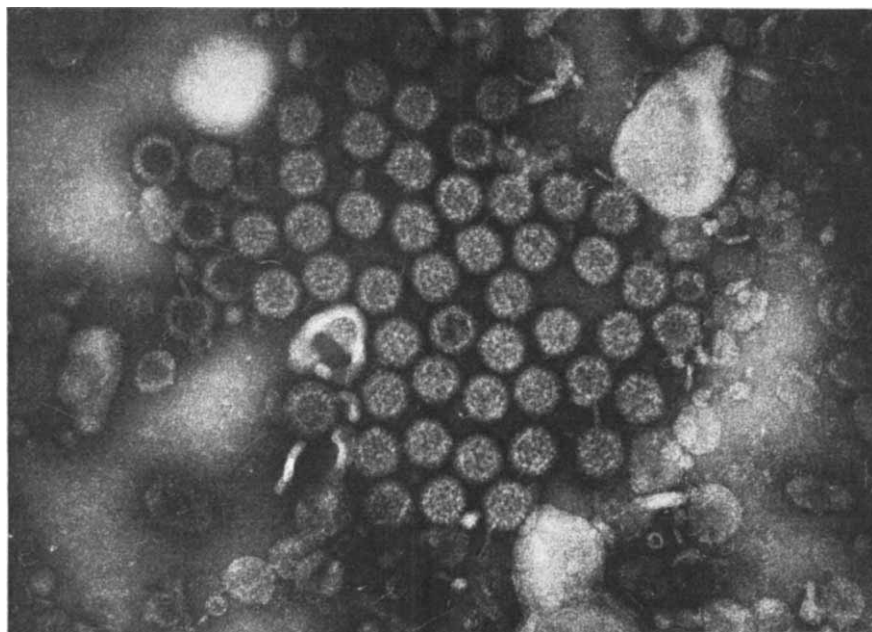
# The virus of acute diarrhoea

from Anthea Thornton  
and Arie J. Zuckerman

ACUTE infectious gastroenteritis has long been one of the commonest causes of childhood illness throughout the world. In England and Wales (1961) acute diarrhoea accounted for 6% of deaths in the first year of childhood. But in technically underdeveloped countries, where malnutrition and other debilitating diseases are undoubtedly important contributing factors, deaths from acute diarrhoeal disease in early life are of such magnitude as to be a leading cause of mortality. Probably no more than an eighth of the infants and children of the world are free from appreciable risk of a lethal diarrhoea. Some episodes of gastroenteritis are clearly associated with certain bacterial pathogens; in at least 65% of cases, however, such agents cannot be isolated. Viruses of various type have occasionally been isolated but in most cases their pathogenic role has remained unestablished.

Recently a new candidate virus, originally classified as a member of the orbivirus group, was seen in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis (Bishop *et al.*, *Lancet*, ii, 1281–1283; 1973) and subsequently in negatively stained faecal extracts from such children (Flewett *et al.*, *Lancet*, ii, 1497; 1973). Morphologically similar particles have since been detected in duodenal fluids and faecal extracts by numerous other workers. Throughout the world, these viruses have now been reported in 396 out of a total of 827 (48%) of children with acute enteritis, and in only two of 357 children without infection. They have rarely been detected in children over the age of six, and are more often encountered in winter. There is now convincing evidence that the new virus is an aetiological agent in acute childhood gastroenteritis. The virus infects epithelial cells only during the symptomatic stage of the illness and its presence coincides with histological abnormalities and depressed duodenal mucosa disaccharidase levels. Seroconversion has been demonstrated in some children and oral transmission of infection has been achieved with one adult volunteer.

The virus differs from both the reovirus and orbivirus group in serology, morphology and polypeptide composition. The virions consist of a core about 38 nm in diameter, bounded by a membrane from which short cylindrical capsomeres radiate outwards like the spokes of a wheel. An outer layer of capsid subunits seems to be attached to the tops of the inner ones; they are



The characteristic morphological appearances of the 'rotavirus' in negatively stained faecal extract of an infant with acute gastroenteritis.  $\times 126,000$ .

continuous at the periphery of the virions giving the impression of a continuous membrane surrounding the virus (intact diameter 60–65 nm). The wheel-like appearance of these viruses (see figure) prompted the suggestion that they be called 'rotaviruses' (Flewett *et al.*, *Lancet*, ii, 61–63; 1974), though Davidson *et al.* (*Lancet*, i, 242–246; 1975) now suggest that 'duoviruses' may be more appropriate.

Any new genus eventually proposed would probably also include the morphologically identical viruses that cause epidemic diarrhoea of infant mice (EDIM) and some outbreaks of neonatal calf and pig diarrhoea, the simian SA11 virus and the O agent isolated from gut washings in sheep and cattle. Complement fixation and immunofluorescent tests show that the human virus, the EDIM virus, and the Nebraska calf diarrhoea virus are also related antigenically. Immune electron microscopy revealed that human convalescent sera reacted with the human and calf viruses, both the intact particles and those which had lost their outer capsid layer, whereas calf antibody reacted only with the inner capsid layer of the human particles. Thus it seems that the inner capsid antigens are the common group antigens detected by complement fixation and immunofluorescent tests and the outer capsid antigens are probably type-specific. This view is reinforced by the finding that the human viruses do not infect calves.

The calf, pig and EDIM virus have been grown in tissue culture and lately the human virus has been shown to replicate in foetal intestine organ culture. Moreover immunofluorescent

studies showed that the virus-specific antigen was localised in the epithelial lining of the villi (Wyatt *et al.*, *J. infect. Dis.*, 130, 523–528; 1974) which supports the earlier duodenal biopsy findings of Bishop *et al.* (1973). In the future the development of techniques by which human rotaviruses may be propagated to high titres in all cultures may eventually lead to development of a vaccine. An orally administered attenuated vaccine effective against the calf diarrhoea virus has already been prepared.

No other viruses have conclusively been shown to cause gastroenteritis in older children and adults. Parvoviruses (22 nm, DNA viruses) may cause bovine enteritis and similar viruses have been found by immune electron microscopy in stools from patients with gastroenteritis. They seem, however, to be found as easily in patients without gastroenteritis. Slightly larger (27 nm) picorna-like viruses have been detected in faecal extracts from an outbreak of gastroenteritis in Norwalk, Ohio. Transmission of infection and the detection of antibody to the Norwalk agent in infected people by immune electron microscopy have also been achieved. But the similarity of such small isometric particles to bacteriophages necessitates extreme caution in the interpretation of the findings in the electron microscope, and the specificity of virus-agglutinating reactions in immune electron microscopy studies has yet to be confirmed. In pigs and calves coronaviruses are also an important cause of enteritis though no cases have yet been reported implicating this group with human gastroenteritis.

Overall, existing evidence points to rotaviruses as a most important cause



of infantile gastroenteritis throughout the world. It is important to note, however, that to date few specimens have been examined from regions where gastroenteritis mortality rates are particularly high. The use of the electron microscope for rapid and reliable detection of rotaviruses in clinical specimens and the development of serological methods of identification will greatly facilitate further studies.

## Does Hex A depend on Hex B?

from a Correspondent

THE question of the relationship of the N-acetyl hexosaminidases is still in dispute. It has been known for six years or so that two major electrophoretic forms of hexosaminidase (A and B) occur in human tissues and also that one of these (Hex A) is deficient in patients with Tay-Sachs disease, a fatal recessive disorder of infants characterised by the accumulation of a complex lipid, the ganglioside GM<sub>2</sub>, the *in vivo* substrate of the enzyme, in the brain. The deficiency of both enzymes is also found in some patients—a variant which is often called Sandhoff's disease. It has generally been thought to be unlikely that two separate and independent gene loci code for the hexosaminidases, as the deficiency of both A and B in Sandhoff's disease would presumably require two separate mutations (at the locus for A and for B). The deficiency of Hex B alone has never been reported and Tay-Sachs disease is extremely rare—thus the coincidence of the two homozygous mutant genes in the same individuals would be expected to be very rare indeed. But twenty or so cases have in fact already been reported.

Biochemical and immunological evidence suggests that the enzymes are closely related, and two types of model have been proposed for their relationship. One is that Hex A and B possess similar and dissimilar subunits (for example A= $\alpha\beta$ ; B= $\beta\beta$ ). The other is that A and B are essentially the same protein, one enzyme being a secondarily (possibly enzymatically) modified form of the other.

In theory one might expect to learn something from the study of somatic cell hybrids. Human-rodent hybrid cells progressively lose their human chromosomes. By analysing the hybrids in a search for the coincident presence or absence of human enzymes and chromosomes genes can be assigned to particular chromosomes. When more than one gene (situated on more than one chromosome) is required to code for a particular protein or proteins the

number of independently located genes involved can be determined.

Information of this kind has been obtained for hexosaminidase but the results from different laboratories are conflicting (van Someren *et al.*, *Human-genetik*, **18**, 171, 1974; Lalley *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1569, 1974; Nguyen Van Cong *et al.*, *C.r. Acad. Sci. Paris.*, **278**, 1761, 1974; Gilbert *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 263, 1975). In the most recent paper on the subject Gilbert *et al.* claim that there are indeed two independent gene loci that code for the hexosaminidases. The gene for Hex B is assigned to chromosome 5 and the gene for Hex A (in agreement with the other groups) is shown to be on the same chromosome as mannose phosphate isomerase (MPI), which has now been assigned to chromosome 15 (van Heyningen *et al.*, *Ann. Hum. Genet.*, **38**, 295, 1975). This result differs from that obtained previously by Lalley *et al.* and Nguyen Van Cong *et al.* These authors never observed the retention of human Hex A in the absence of Hex B in the hybrid cells and suggested that Hex A requires the presence of two human genes for its expression and B requires only one of the two genes (which now seems to be on chromosome 5). These findings were consistent with both the favoured models for Hex A/Hex B relationship. The lack of observation of one particular combination is not however in itself very convincing evidence for a dependence model. The best supporting evidence would come from clones which have MPI but neither Hex A nor Hex B, predicted from this model for those clones which have chromosome 15 but not 5. Very few definite clones of this type have so far been reported. Gilbert *et al.* on the other hand claim to have clones of all the possible types (including Hex A+, Hex B-), which are consistent with their model. There are, however, some major problems that cast doubt on the interpretation of their results.

In order to avoid implicating two mutations in Sandhoff's disease, Gilbert *et al.* have suggested that the defect may be a mutation in a locus which controls the expression of both A and B or makes an activator. The first problem is that there is some evidence that certain Sandhoff patients at least make non-active antigenically cross reactive 'hexosaminidase' proteins (Srivastava and Beutler, *J. biol. Chem.*, **249**, 2054, 1974; Carroll and Robinson, *Biochem. J.*, **137**, 217, 1974). If the control model were right then no such protein should be synthesised. Second, another informative hexosaminidase variant has been reported in a healthy parent of a patient with Sandhoff's disease (Dreyfus *et al.*, *New Eng. J. Med.*, **292**, 61,

1975). This individual has a deficiency of both Hex A and Hex B, as detected using an artificial substrate, and is probably heterozygous for the Sandhoff allele and one which produces kinetically abnormal A and B enzymes (which seem to hydrolyse the natural but not the artificial substrates for the enzyme). This variant strongly suggests that the enzymes must have polypeptides in common.

From the technical point of view, other workers have commented on difficulties involved in identification of the human hexosaminidase components in hybrid cells, and it may be that a problem of this kind is leading to misclassification of the enzymes.

So, the argument goes on . . .

## Genes, enzymes and behaviour

from T. J. Crow

THAT heredity is a powerful determinant of behaviour was first cogently argued in Lange's book *Crime as Destiny* (Allen and Unwin, London, 1931). The controversy about the genetic contribution to intelligence has recently had a lively renaissance; H. J. Eysenck, a protagonist of the innate factor school, has also championed the view that there are important hereditary influences on the personality variables neuroticism and extraversion. In the more limited field of overt psychiatric disease the contribution of genetic factors to the major psychoses, schizophrenia and manic-depressive illness, is now generally accepted and has recently been emphasised by studies on adopted offspring of schizophrenic patients (for example Heston, *Science*, **167**, 249; 1970; Kety, *Am. J. Psychiat.*, **131**, 957; 1974). Evidence for genetic factors in neurotic illness (for example Shields, *Monozygotic Twins*; Oxford University Press, 1962) is less well-known, but perhaps equally significant.

Attempts to study the links between genes and behaviour have led to the development of animal models. In selective breeding experiments in rats Broadhurst (*Nature*, **184**, 1517; 1959) was able to demonstrate a genetic influence determining 'emotionality', as defined by increased defaecation rates in an open field apparatus, and an independent factor of 'extroversion-introversion' (*J. exp. Psychol.*, **56**, 349; 1958).

Bovet and his colleagues (*Science*, **163**, 139; 1969) demonstrated very striking differences between strains, and consistencies within strain, in the rates, and temporal patterns, of acquisition of a conditioned avoidance response in mice. Such experiments invite specula-

tion concerning the neurochemical basis of the differences, and, in view of evidence for an important role for central monoamine neurones in behavioural control, focus interest on genetic variations in mechanisms regulating monoamine turnover.

Recently Ciaranello, Lipsky and Axelrod (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 3006; 1974) have reported that the concentrations of three adrenal catecholamine synthesising enzymes, tyrosine hydroxylase, dopamine- $\beta$ -hydroxylase and phenylethanolamine N-methyl transferase, are twice as high in the adrenals of Balb/cJ mice as in those of the related inbred strain Balb/cN, and that heterozygous progeny are intermediate between their parents in the concentrations of these enzymes. Ciaranello *et al.* (*J. biol. Chem.*, **249**, 4528; 1974) examined the F<sub>1</sub>, F<sub>2</sub> and backcross progeny of the mating between these sublines and concluded that single gene loci control the steady state concentrations of each enzyme. The correlation between the activities of these enzymes suggested either that the loci were linked structural genes or that a single regulatory gene locus controls the phenotypic expression of the three enzymes. Ciaranello *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 3006; 1974) also draw attention to differences between the two strains in fighting behaviour after a 2-week isolation period. In this case analysis of the F<sub>2</sub> mice suggested the behavioural differences were determined by a single gene with fighting recessive.

Such genetically determined behavioural differences could be coincidental, a cause, or a consequence, of the altered enzyme levels, but evidence for a relationship between aggressive behaviour and central catecholamines (Scheel-Krüger, Randrup, *Life Sci.*, **6**, 1389; 1967; Welch and Welch, *Commun. Behav. Biol.*, **3**, 125; 1969) may be relevant to the association. On the other hand much recent evidence supports the hypothesis that there is an association between central catecholamine-containing neurones and the mechanisms of reward (Crow, *Psychol. Med.*, **2**, 414; 1972; Ritter and Stein, *J. comp. Physiol. Psychol.*, **85**, 443; 1973). The question of whether unitary neurochemical mechanisms are associated with single dimensions in behaviour is one which increasingly will have to be approached.

On the opposite side of the coin is an accumulation of evidence for environmental influences on central catecholamine turnover (Thierry, Javoy, Glowinski and Kety, *J. Pharmac. exp. Ther.*, **163**, 163; 1968; Lewy and Seiden, *Science*, **175**, 454; 1972). Such findings may illuminate how genetic and environmental influences interact in the pathogenesis of the psychoses.

## A plasmid dissected

from David Sherratt

A NUMBER of features has made the bacterial plasmid Col E1 an increasingly attractive DNA molecule for studies of genetic organisation, DNA replication and gene expression. Its small size of  $4.2 \times 10^6$  daltons, sufficient to code for about seven average-sized proteins, makes it easy to isolate and handle in analytical studies and its replication can conveniently be studied both *in vivo* and in a soluble *in vitro* system. In addition, the Col E1 molecule has a single unique site for double strand cleavage by the sequence specific restriction endonuclease *ecoRI*. This provides an invaluable positional reference for structural and replication studies of Col E1 and also provides an ideal tool for *in vitro* construction of hybrid DNA molecules containing DNA from either pro- or eukaryotes covalently linked to Col E1 (see for example Herschfield *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3455–3459; 1974).

Localisation and analysis of specific regions of DNA (such as replication origins, strand interruptions, specific protein binding sites) by electron microscopy or other techniques requires some reference point. In linear molecules with unique ends such as bacteriophage T7, the ends provide reference points (with ambiguity unless the ends are distinguishable). But Col E1 and many other DNA molecules of interest are either circular or like phage T4 have non-unique ends. Various approaches have been used to provide visual markers in such molecules. Inman (*J. molec. Biol.*, **18**, 464–476; 1966) used mild denaturing conditions to visualise the AT-rich regions of phage lambda as 'loops' which occurred at fixed unique positions. This technique has been widely used, though it is inapplicable for many molecules as small as Col E1. Another approach has been to construct heteroduplexes *in vitro* between molecules differing by a deletion or addition of genetic material (see Sharp *et al.*, *J. molec. Biol.*, **71**, 471–497; 1972), though this technique is not easily applicable to analysis of replicating molecules. The use of sequence specific restriction nucleases such as *ecoRI* to introduce double strand breaks at specific sites in DNA is a relatively simple technique and is being extensively used in the physical mapping of small DNA molecules.

Replicating Col E1 molecules have been isolated from exponentially growing *E. coli* cells, by Lovett, Katz and Helinski (*Nature*, **251**, 337–340; 1974), from *E. coli* minicells by Inselberg (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 2256–2259; 1974) and from a soluble *in*

*vitro* system which allows Col E1 initiation, semiconservative replication, termination and ligation to give complete closed circular duplexes (Tomizawa *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 802–806, 1403–1407, 2260–2264, 4935–4939; 1974). In each of these systems, the majority of replicating molecules when examined by electron microscopy are seen to be circular with two branch points; such *theta* structures are characteristic replicative intermediates of many circular replicons. The positions of the branch points with respect to the *ecoRI* cleavage site were determined independ-



### THE FATAL BALLOON ASCENT

THE readers of NATURE are no doubt aware of the fatal result of the recent ascent of the balloon *Zenith*; the following authentic details at first hand will no doubt be of interest:—

CIRON (Indre), April 17.

The *Zenith* was sent up on the 15th of April in order to determine the quantity of carbonic acid contained in the atmosphere at an altitude of 24,000 feet. The "let go" was given at twenty-five minutes to twelve A.M. The captain was M. Sivel, and there were only two passengers, M. Gaston Tissandier and M. Crocé-Spinelli. The ascent took place gradually in a slight E.N.E. wind, the sky being blue but vaporous. The rate of ascent was calculated to be nine feet per second, but diminished gradually. Shortly after one o'clock the altitude obtained was 22,800, and the passengers were quite well, although feeling weak. The inhalation of oxygen produced good restorative effects when tried. Then a consultation took place, and the *Zenith* being in equilibrium, a quantity of ballast was thrown overboard. M. Tissandier then fainted, and is ignorant of what was felt by his friends.

At eighteen minutes past two he was awakened by M. Crocé-Spinelli warning him to throw over ballast as the balloon was fast descending. He obeyed mechanically, and at the same time Crocé-Spinelli threw overboard the aspirator, weighing eighty pounds. Tissandier then wrote in his book a few disconnected words, and again fell asleep for about an hour. When he awoke, the balloon was descending at a terrific rate; no more ballast was left to be thrown away, and his two friends were suffocated. Their faces had turned black, and the blood was flowing from their mouth and nose. They were evidently dead. It was a terrible situation.

from *Nature*, **11**, 495; April 22, 1875



ently by each of the above groups of workers. In each case, the distance from the cleavage site to the nearest branch (for molecules less than 60% replicated) was shown to be about 18% of the genome length, while the length from the other branch was variable and inversely proportional to the extent of replication of the molecule. These results show that replication is initiated about 18% of a genome length from the *ecoRI* cleavage site and proceeds unidirectionally away from that site. Whereas molecules in all stages of replication were observed *in vivo* and *in vitro*, molecules isolated from the *in vitro* system in the presence of glycerol (10%) and spermidine (2 mM) were nearly all about 7% replicated, indicating that there is a potential rate-limiting step at this stage of replication (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 2260–2264; 1974).

Two other structural features of Col E1 molecules have also been examined with respect to the *ecoRI* cleavage site. Col E1 molecules can be isolated as supercoiled 'DNA-protein relaxation complexes' which undergo a strand-specific single-strand cleavage after treatment with protein denaturing agents. Helinski and co-workers (*Nature*, **251**, 337–340; 1974) first showed that the break in such molecules is located about 18% of the genome length from the *ecoRI* restriction site, that is, either at the origin/terminus of replication or at an equal distance the other side of the restriction site. To distinguish between these possibilities Tomizawa *et al.* (*Nature*, **253**, 652–654; 1975) tested the ability of pulse-labelled newly initiated Col E1 DNA to hybridise with Col E1 molecules in which the DNA in the region of the break in 'relaxation complex' had been removed but which still contained the sequences present in the symmetrical position 18% of a genome's length the other side of the *ecoRI* site. The newly initiated DNA failed to hybridise to such molecules, showing that the break in 'relaxation complex' is coincident with the origin/terminus of replication and reinforcing the suggestion that relaxation complex is involved in Col E1 replication. Col E1 supercoils containing a single stretch of RNA in either strand can be isolated after extensive replication in chloramphenicol (Blair *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2518–2522; 1972). Tomizawa and co-workers then went on to analyse the position of the gap created by removal of the RNA with respect to the *ecoRI* cleavage site and showed the RNA to be located at random, indicating that it is not a unique piece of RNA involved in initiation of DNA synthesis at the origin, though it could be involved in priming of 'Okazaki fragment' synthesis or

merely an artefact introduced during chloramphenicol replication.

This type of experiment can be extended to analyse the organisation and control of Col E1 genes. For example, the polarity of the 'nicked' strand in 'relaxation complex' with respect to the *ecoRI* site has been determined by Tomizawa (*Nature*, **253**, 652–654; 1975) and it is also known that Col E1 hybrid molecules with other DNA inserted at the *ecoRI* site fail to synthesise colicin yet remain immune to colicin killing (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 3455–3459; 1974), suggesting that the immunity gene does not cross the *ecoRI* site, yet the colicin gene or one required for its synthesis does. Using other restriction enzymes and an *in vitro* protein synthesising system it should be possible to map the Col E1 genes, isolate the gene products and study their regulation and functions.

## Quantising gravity?

from John Taylor

RECENT developments in unifying the forces of nature have been proving successful but so far have not involved gravity. The quantum mechanical nature of the other three forces has up to the present prevented gravity from being treated alongside them due to great difficulties in setting up a sensible quantum theory of gravity. Yet the problem of quantising gravity is a pressing one if a unified theory of all the forces of nature is to be obtained. There is also the need for a quantised theory of gravity to describe the first  $10^{-43}$  seconds of the big bang and to prevent the annihilation of matter at the centre of a black hole.

These and other reasons have still left a great deal to be desired in the earlier programme of quantising gravity. In the last three years great strides have finally been made by using techniques developed to handle the unified theories of weak and electromagnetic interactions. This has proved possible since both unified models and gravity are invariant under gauge groups, these being internal symmetries in the former case and the space-time coordinate transformations in the latter. The perturbation rules for calculating scattering matrix elements so as to be unitary at each order were developed in the 1960s for theories invariant under quite general gauge groups. Unphysical modes of the gauge fields were found to be required to preserve unitarity, though these modes could never be created so never caused embarrassment.

The recent advances have stemmed from attempts to obtain sensible answers for the resulting perturbation

calculations in the gravitational case. The outstanding question has been of the removal of the ultraviolet divergences (arising from particles of arbitrary high energies in internal states) which plague any realistic quantised field theory. In the unified weak and electromagnetic models these ultraviolet divergences can be removed completely by absorption into and redefinition of the masses and charges of the particles present: the renormalisation programme. Quantised gravity is not explicitly renormalisable in the sense that the ultraviolet divergences cannot automatically be removed by redefinition of particle masses. The recent progress in quantum gravity is in evaluation of some of these ultraviolet divergences and their removal by additional terms (counter terms) in the action function of the fields.

The first step in this programme was taken by 't Hooft and Veltman (*Annls Inst. Henri Poincaré*, **20**, 69–94; 1974) following the pioneering work of de Witt (*Phys. Rev.*, **162**, 1195, 1239; 1967) who evaluated for pure gravity the counter terms that eliminate the ultraviolet divergences of all Feynman diagrams with a single loop of internal particles (in this case only gravitons). They showed that S-matrix elements from such single-loop diagrams were finite after suitable field renormalisations had been performed; no infinite counter terms were needed. But no such tricks were discovered which eliminated single-loop diagram divergences in the case of gravitation interacting with a scalar field, so that such terms were needed to be added to the action in such a way as to change it considerably.

This programme of investigating if infinite counter terms are needed to make single-loop S-matrix elements finite has been extended to more realistic forms of quantised matter fields interacting with quantised gravity. Deser and Van Nieuwenhuizen (*Phys. Rev.*, **D10**, 401–420; 1974) have shown that extra counter terms are needed in the case of otherwise free photons or fermions, and a similar situation holds for charged mesons in self-interaction as well as gravitation, as it does if photons are also involved (Nouri-Moghadam and Taylor, *Proc. R. Soc.*, in the press). This latter situation is particularly appropriate since it allows for some of the subtleties of the unified weak and electromagnetic interaction models.

It could well be that the contribution of diagrams with higher numbers of closed loops of internal particles could cancel the infinities of the single loop ones. However using the modified action function, with single loop counter terms included, generates two new particles. One of these is a scalar

particle which can be made so massive as to put it beyond observation; the other, of spin two, destroys the theory since it is a ghost particle with a negative norm. As such it violates unitarity, and certainly in particle physics ghosts should frighten one if they can be seen. If this spin-two ghost particle could be decoupled from all other fields then it would have been rendered harmless, though recently it has been realised that such decoupling is very difficult for gravity in the presence of photons or scalar or spin- $\frac{1}{2}$  particles.

If such is the case there are two ways forward in quantising gravity. One is to start afresh from an alternative beginning to that of Einstein; this is also very difficult since there are almost unavoidable arguments which lead to Einstein's theory of gravity (Deser, *J. gen. Rel. Grav.*, 1, 9-18; 1970). The other alternative is to investigate more carefully the forms of matter for which there is cancellation of the ultraviolet divergences from single loop diagrams. This is a difficult but not impossible problem. Its solution, if there is one, will give a hint as to the form of quantised matter-gravity interaction which is infinity free at all orders; it is to be hoped that there is only one such solution. If there is no such form of theory then gravity will be different indeed from the other forces of nature.

## Alpha emission in fast pion reactions

from P. E. Hodgson

MANY researchers in recent years have provided evidence that nucleons in the region of the nuclear surface tend to condense into alpha particles. This naturally affects the cross sections of reactions involving the transfer of a cluster to or from a nucleus, and it is by analysis of data on such reactions that an estimate of the alpha-clustering probability can be obtained.

One of the most direct ways of demonstrating the existence of alpha particles on the nuclear surface is to knock them out with a suitable projectile. Unfortunately the cross sections of these reactions are quite low so that thick targets must be used, and this stops most of the alpha particles from emerging. Instead of trying to detect the alpha particles directly, the residual nuclei after the reaction had taken place were identified from their characteristic gamma-ray spectra. It was then found that a substantial proportion of these nuclei were just the target nucleus less one, two or three alpha particles. This strongly suggested that alpha particles were ejected from the nuclei by

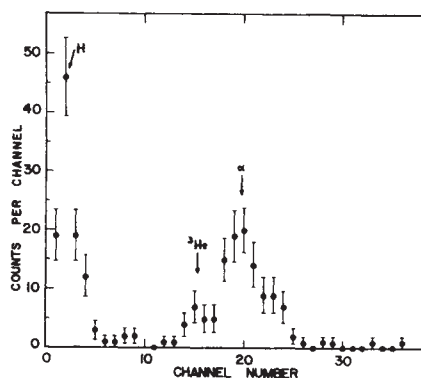


Fig. 1 Particle spectrum showing alpha particles and helions.

the projectiles, though of course the possibility that the nucleons were ejected individually could not be excluded.

This result was found in many experiments using fast and slow kaons and pions as projectiles (*Nature*, 249, 616; 1974).

Some doubt was thrown on the interpretation of these results by an alpha particle knockout process when it was found by statistical model calculations that the observed distribution of final nuclei is what might be expected on purely energetic grounds. It therefore became of great importance to demonstrate the reality of the alpha knockout process by detecting the emitted alpha particles directly.

This has recently been done by a group at Saclay and Tel Aviv (*Phys. Rev. Lett.*, 34, 485; 1975). They bombarded  $^{27}\text{Al}$  with 70 MeV negative pions and found a significant cross section for the emission of He ions, predominantly alpha particles. They were able to overcome the problem of low yield by using the intense electron beam from the Saclay linear accelerator that produces a pion beam of intensity  $10^6$  pions per second. This made it possible to produce a significant number of alpha particles in a target thin enough for most of them to escape.

The observed particle spectrum at  $90^\circ$  to the beam is shown in Fig. 1 and has a significant alpha peak and possibly a smaller peak due to helions. The energy spectrum of the He ions in Fig.

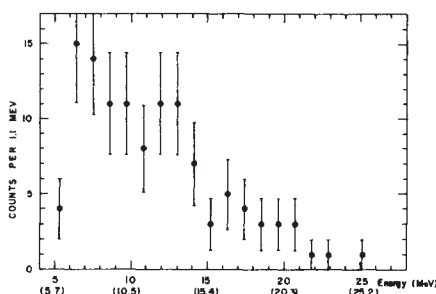


Fig. 2 Energy spectrum of the He ions emitted from the bombardment of  $^{27}\text{Al}$  by 70 MeV negative pions.

2 shows that their intensity has a maximum at about 10 MeV, and falls to zero around 25 MeV. The differential cross section for alpha-particle emission is  $6.2 \text{ mbarn sr}^{-1}$  in the energy range 5.5-30 MeV.

Calculations within the framework of a cascade evaporation model indicate that such a yield of alpha particles can be explained by simple evaporation following the excitation of the nucleus by a series of pion-nucleon interactions. Further work is now needed, particularly measurements in the forward direction to see if any of them can be attributed to a direct knockout of pre-existing alpha particles on the nuclear surface.

## Electric seaweed eggs

from Julian Lewis

MANY non-spherical embryos develop from spherical eggs. What then is the origin of their asymmetry? In most cases the fertilised egg, despite its spherical shape, does not have spherical symmetry in its chemical constitution: from the outset there is some local specialisation of the membrane or cortex, or an uneven distribution of material in the cytoplasm. But there is no need in principle for an egg to start life lopsided. It may have at first true spherical symmetry, and lose this through the development of an instability as it matures. Its internal dynamics may be such as to magnify the slightest departure from symmetry into a full-blown polarisation. The egg would thus spontaneously become polarised, but the direction of its polar axis would be subject to control by almost any sort of weak environmental vector.

Jaffe and his colleagues have studied the eggs of the closely related seaweeds *Fucus* and *Pelvetia* (*Advances in Morphogenesis*, 7, 295; 1968). These eggs, about  $100 \mu\text{m}$  in diameter, are at first spherical. Within a few hours after fertilisation they glue themselves to the sea bed, and then germinate: they develop a bulge and become pear-shaped as the future rhizoid begins to grow out. Practically anything, from a beam of light to a gradient of pH or a mechanical deformation, can govern the direction in which the rhizoid emerges. This is strongly symptomatic of a symmetry-breaking instability; some other conceivable interpretations can be ruled out experimentally (Jaffe, *Science*, 123, 1081; 1956).

Thus interest centres on the positive-feedback mechanism in the egg which amplifies and maintains whatever asymmetry happens to arise. Jaffe suggests that a sort of self-electrophoresis



may occur: if one part of the cell membrane transports ions at a different rate from the rest, a steady electric current may be driven through the cell, carrying towards the specialised patch of membrane just those charged molecules which support its specialisation. This speculation has some experimental backing.

In the first place, Jaffe (*Proc. natn. Acad. Sci. U.S.A.*, **56**, 1102; 1966) aligned about 200 eggs, similarly polarised, in seawater in a loose-fitting capillary tube. He found a potential difference of about 40  $\mu$ V between its ends, and argued persuasively that this must arise from a flow of current, with an estimated density of 6  $\mu$ A  $\text{cm}^{-2}$  at each rhizoid tip. This might plausibly be driven by a cellular e.m.f. of the order of millivolts.

More recently, Nuccitelli and Jaffe have described in detail (*J. Cell Biol.*, **63**, 614; 1974) a probe small and sensitive enough to measure the electric fields and hence the current densities within 50  $\mu$ m of a single egg (*Proc. natn. Acad. Sci. U.S.A.*, **71**, 4855; 1974). The tip of the probe vibrates (at about 200 Hz), and picks up the minute change of potential ( $10^{-7}$  to  $10^{-8}$ V) between the extreme points of its path, typically 30  $\mu$ m apart. The findings roughly match the results of the earlier work. A striking phenomenon is the occurrence of large irregular spikes, lasting about a minute, during which the signal from the probe increases by a factor of ten or a hundred. These spikes may well mark the insertion of fresh vesicles of membrane at the growing rhizoid tip; though sceptics might say they could be artefacts—transient diffusion potentials, perhaps, due to polyelectrolytes released from the egg and turbulently stirred about by the vibrating probe.

Before the rhizoid axis becomes irreversibly determined at germination, there is an impressionable phase when environmental cues, such as light, can impose on the eggs a tentative and still reversible polarity. Robinson and Jaffe (*Science*, **187**, 70; 1974) took a sieve with small round holes, plugged each hole with a *Pelvetia* egg at the impressionable stage, shone a light from one side, and measured the rates of entry and exit of radioactive calcium ions through the dark (prospective rhizoid) and light (prospective thallus) ends of the eggs. The rate of entry on the dark side was at first about three times greater than on the light side, and the rate of exit about three times less. The eggs, therefore, must pump calcium through themselves. This pumping of calcium peters out towards the time of germinating, just as the flow of electricity begins.

One still does not know how strongly the ionic currents affect the cytoplasm,

or whether the cells are in fact polarised by self-electrophoresis. But the evidence for persistent currents through fucoid eggs is important, if only in drawing attention to a factor in cell organisation which tends to be neglected, more because it is hard to measure than because it is insignificant, rare, or peculiar to seaweeds. One might here perceive a distant echo of the finding, for instance, that the localisation of acetylcholine sensitivity in muscle cell membranes depends on electrical activity (Lomo and Rosenthal, *J. Physiol., Lond.*, **221**, 493; 1972).

## Between Earth and Sun

from L. J. C. Woolliscroft

A MIST (Magnetosphere, Ionosphere and Solar-Terrestrial Relations) Meeting, arranged by the Royal Astronomical Society, was held at the University of Exeter on March 25 and 26.

THE significant role of atomic oxygen was stressed in a session on the lower ionosphere. M. R. Bowman and L. Thomas (Appleton Laboratory, Slough) gave two reasons for studying the density of oxygen: first, it breaks the chain of reactions which starts with electrons and ends with negative ions, and thus it tends to prevent the loss of free electrons. Second, it also breaks another chain by which positive ions such as  $\text{N}_2^+$  react to form the series of water cluster ions,  $\text{H}^+(\text{H}_2\text{O})_n$  where  $n$  can be at least as high as seven. These cluster ions dominate the ion spectrum below about 85 km and it was encouraging to see that the model of Bowman and Thomas predicts an increase in atomic oxygen above this height with a diurnal variation below it. P. H. G. Dickenson (also Appleton Laboratory) presented new rocket measurements of atomic oxygen using the resonant fluorescence and absorption techniques. These methods give better results than measuring the resistivity of thin silver films (oxidised by the atomic oxygen) because the response is faster. His results seem broadly to agree with the Bowman and Thomas model but as yet the whole diurnal variation has not been studied.

F. N. Byrne (Queen's University, Belfast) showed some preliminary data on the ionised and neutral concentrations of magnesium, aluminum and iron. The ionised form of the metals has been shown to be important at altitudes above those characteristic of the cluster ions (typically 90–110 km) but the ratios of the ionised to neutral concentration is uncertain. The reaction

of neutral metal atoms with nitric oxide ions to give metal ions should be very efficient and so some reverse mechanism is needed to explain Byrne's finding that significant densities of neutral atoms remain. He suggested this was another role for atomic oxygen.

The solar magnetic field and its effects are now felt to be more complicated. W. M. Glencross (University College, London) challenged the conventional theory for the heating of the solar corona by shock waves. He suggested that magnetic fields also play a part and showed Skylab and X-ray photographs to illustrate that the intersecting loop field configurations he proposes can in fact exist. G. M. Simnett (Birmingham University) invokes the magnetic field at a few solar radii to account for the delays (at least 20 minutes) in the propagation of energetic protons and electrons from solar flares. This process also accounts for modification of the energy spectrum of the particles during their passage through the corona which can be inferred from measurements near the Earth.

The yields of turnips and other crops were discussed, together with the whole relationship between solar phenomena and the weather on Earth, by J. W. King (Appleton Laboratory). The correlation between various climatic and related phenomena, such as rainfall, surface pressure and temperature, with the 11-year sunspot cycle is interesting enough, but what is much harder to envisage is why some properties correlate with the 22-year solar cycle of field reversals. Perhaps the most striking of these are the periods of drought in the United States which at least in part led to the depression of the 1930s, and the mini-depression of the 1950s. On a much shorter time scale the weather is altered by magnetic storms and after reversals of the magnetic field of the solar wind at the magnetosphere.

These results were greeted with scepticism by a meteorologist in the audience who wanted details of a mechanism rather than King's qualitative suggestions of various possibilities including variations in the solar constant (the power emitted by the Sun), solar protons leading to water molecules in the atmosphere, and perturbations of the atmosphere at, say, 100 km causing a resonance in the lower atmosphere.

D. M. Willis (Appleton Laboratory) presented results of how the magnetosphere might be involved in some form of coupling between the solar wind and the weather. On the basis of the energy available it does not seem that the magnetosphere can provide the mechanism unless it is by some triggering or resonance phenomenon.

# review article

## Computer programming as a cognitive paradigm

P. J. Hayes\*

*As work on artificial intelligence has made increasingly clear, intelligent behaviour depends more on an organised knowledge of the real world than on problem-solving mechanisms. This has led in artificial intelligence research to an increasing preoccupation with techniques for representing such knowledge, and recently to a view of programming as itself a form of knowledge representation.*

As an essential part of the methodology of artificial intelligence research, the art and science of writing computer programs has inevitably been a central concern. It has been suggested that artificial intelligence should be regarded as a science of which experiments are programs. In the past few years, however, programming has become a focus of attention in its own right, and researchers have begun to look at the activity of writing programs as itself a problem area to which artificial intelligence might be applied, as it has already been applied to chess playing, recognising speech, or designing a bathroom. Systems are now being designed and implemented to write programs to solve a given problem, which 'debug' programs<sup>1</sup>, and which reason about the properties of programs. Moreover, the development of the field since 1969 has been marked by a rash of new programming languages which represent a distinct break with the main computing tradition. At the same time, both in Britain and in the USA, the main funding agencies (the Science Research Council and the Advanced Research Projects Agency), have noted the emergence of "automatic programming" as an important growth area in artificial intelligence.

This apparently narcissistic concern with programming reflects a fundamental change in our view of what sort of knowledge has to be stored and used by an intelligent program, and how it can be represented. The advance has emerged, characteristically, from a series of acrimonious debates, starting with a methodological dispute and finishing with a technical synthesis in which both sides can participate without losing face. In this article I want to sort out a little of what is now agreed and what is not, and what consequences the new ideas have, both for our view of intelligence and for the development of programming. To begin, however, we must be quite clear what we mean by 'a program'.

### Meaning and procedure

Asked to define 'program', most people would probably come up with something like 'sequence of instructions for a computer'. This corresponds to the usual layman's view of the computer: an electronic device which obeys long sequences of simple unambiguous instructions, very quickly, without errors. But this definition would be adequate only for programs written in machine codes, which bear a direct relationship to the electronics which implement the instructions. Most programs are written in much 'higher level', programming languages, such as ALGOL. Programs in these languages are not simply sequences of instructions, but can have a complex syntactic structure. More importantly, the meaning of a programming language is not

directly related to the structure of any particular physical computer.

The use of the term 'level' here refers to a fundamental computing idea. An ALGOL programmer, for example, can ask for, say, a two-dimensional array of numbers. When his program is run, complicated machine-code instructions will be generated which simulate this structure. There are many ways of doing this: which way is used is completely invisible to the ALGOL programmer. It is a decision at a lower level than that of the ALGOL program. A primitive operation (creating or using an array) at the higher level corresponds to an elaborate mechanism at the lower, the workings of which are invisible from above. The ALGOL programmer may himself have in mind that his array represents the floor plan of a cathedral: this, again, is an idea at a still higher level. For, he could have used a wide variety of different data structures, (each probably with several ways of being implemented in code) to represent his conceptual structure. Much of the art of computing consists in this use of complexity at one level to provide clean structures at a higher level, the meaning of which is independent of the encoding. The development of large programs would be impossible without it.

The meanings of high level programming languages have to be defined in terms of a 'conceptual machine' corresponding to the language. This device may be quite unlike any physical machine: for example, the 'ALGOL machine' is capable of directly evaluating arithmetic expressions, automatically maintains an internal dictionary of symbols in the program, and has a memory arranged as a stack (like a pile of plates) rather than as a sequence of boxes. No physical computer was ever built with any of these characteristics. It is important to realise that ALGOL programs are not merely abbreviations of machine code instruction sequences: they have an existence quite independent of any physical machine. The same ALGOL program can be implemented and run on a variety of quite different actual computers operating on totally dissimilar physical principles (for example, electronic, electromagnetic, fluidic) and yet the program retains its unique identity and meaning through these different incarnations. It has been suggested<sup>2</sup> that a facility be incorporated in a high level language to allow a program to query the hardware of the machine about its input-output peripherals, so that it [the program] can organise its files appropriately for the machines it [the program] happens to find itself in from time to time.

In some cases, the conceptual device underlying a programming language is not much like a machine at all. The language SIMULA, for example, is designed to aid the writing of programs to simulate complicated system of activities, such as a dockyard. To understand a SIMULA program one has to think of a collection of processes being created, running in parallel,

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communicating with one another and altering their behaviour.

The 'machine code' picture breaks down also, when we consider the relationship between high level programming languages and their meanings. These languages do not consist of simple imperatives: they also contain, for example, expressions which require to be evaluated, and declarations which define the meanings of names. Some high level languages, indeed, contain no imperatives at all, although they unambiguously specify computations. LISP, a language widely used in artificial intelligence work, is such a one. In LISP, one defines functions (possibly recursively), and asks for them to be evaluated: the language consists entirely of expressions.

A program, then, need not be in any sense a sequence of instructions for a machine. What all programming languages do have in common however, is a concern with activity, in the sense that they describe processes in some sort of device. We will take this, then, as the definition of 'program': a program is a description of a process. Any organised and constructive way of describing processes can be regarded as a programming language.

### Representing knowledge at the right level

Ever since the beginning of AI work, a central idea has been that in order to behave appropriately in some given situation, a program must have available, or be based on, knowledge of the situation and the circumstances. To play chess, one needs to know a lot about chess-playing; to see a scene, one needs to know a lot about the sorts of object in the scene, about perspective, and about *chiaroscuro*.

If this seems like a platitude, it is one. It amounts to saying that there is no magic key to intelligence: the science-fiction idea of the great mechanical brain suddenly becoming a super-intelligence when the last connection is soldered together, is a nonsense. Paradoxically, the central theme of AI work diminishes the role of machinery, emphasising rather the importance of knowledge and inference. (Platitude or not, however, this approach to understanding intelligence differs profoundly from that which would have us first understand how the brain works, in a neurophysiological sense.)

Not surprisingly, then, many AI workers have been concerned with general ways of representing knowledge; with, we could say, general representational schemes. For example, many people including myself have tried to adapt formal logical notations as vehicles for representing 'common-sense' knowledge of the everyday world: others have emphasised, rather, the importance of such non-deductive modes of inference as the use of analogy and inductive generalisation. For example, Newell has worked on a notation<sup>3</sup> for expressing observations of 'analogical similarity' such as "If you think of a nose as a snout, and feet as trotters, then a man can be thought of as a pig".

Merely having a notation to express knowledge is of no use, of course, unless the system can use the knowledge in some way. Each scheme comes equipped with some processing rules which sanction certain inferences to be made using the notation. Logical calculi have such rules of inference corresponding to logically valid deductions; schemes involving networks use, typically, a form of graph-matching to discover whether one network can be imbedded in another; Newell's analogy system uses an elaborate symbol-substitution mechanism to infer new, more complex, analogies from old ones; and so on.

The information encoded in such a general scheme is at a higher level (in the sense described earlier) than that at which the actual program using the information is written (although such a program will almost certainly be written in a high-level language: remember, there can be many levels). For example, the semantics of the knowledge encoded in the schemes—what it is about—is usually nothing whatever to do with the internal workings of any device, but rather concerns facts about some external environment: chess for a chess-playing program, *chiaroscuro* for a vision program, mathematics for a theorem-proving program, actions and their effect on a world for a robot's planning program. Moreover, one of these schemes

can be implemented differently in different programming languages without altering its meaning (such as by using different data structures to represent a network of relationships).

There have been, and still are, heated debates between different AI schools as to which are the most appropriate vehicles for expressing information about various task domains. It is noticeable, however, that, in practice, no program which has actually achieved an interesting level of performance has ever used any of these methods to represent all the knowledge on which its performance depends.

Many successful programs are simply one-off pieces of code in which the 'knowledge' is embodied directly in the way the details of the program are written. For example (one of many), Kelley<sup>4</sup> wrote a program to find the outline of a person's head in a head-and-shoulders photograph (a police 'mug shot'). This ingenious and successful program was based on a number of facts about heads and their appearance: for example, that heads do not have long narrow projections, that they are more or less symmetrical, that they sit on shoulders; and so on. But these facts were nowhere explicitly represented: rather, the actual program was written in a way which was based on them. For example, the no-projections fact was embodied in a heuristic rule by which the edge-following algorithm refused to follow such contours. The knowledge was encoded at a lower level than that of an explicit representational scheme for expressing such facts.

Even in programs which do use some such general scheme, one often finds that certain kinds of information, crucial to the success of the overall system, are embodied in the lower-level programming details. An early example was Raphael's SIR<sup>5</sup>, a program for answering simple English questions about a data base. I will not describe its linguistic powers, which were rudimentary. Its way of storing information and making inferences, however, was interesting as a pioneer attempt to represent relationships using a network.

The fact: "Hands have five fingers" was encoded by having an arc, from a node called HAND, to a node called FINGER, labelled with the relation HASASPART, and a note to the effect that there are 5 of them. That people have two hands was encoded similarly. If asked how many fingers people have, SIR could make the inference and reply, 'TEN': it did this by using a collection of little programs (written in LISP) which traced along arcs of the network. This sounds quite elegant: but notice that the inference requires also a general fact: roughly, that if an X has N Ys as parts, and a Y has M Zs as parts, then an X has (M times N) Zs as parts. And this fact is not in the network: it is embodied in one of the little LISP programs.

Another more recent example is provided by a much more sophisticated language-understanding system, Schank's MAR-GIE<sup>6,7</sup>. Schank uses an intriguing graphical notation for expressing concepts and facts, which he calls 'conceptual dependency' notation. Many claims are made for the generality of this notational scheme: and yet, when one looks at the details of the system, one finds that the actual inferences are performed by specially-built LISP programs ("inference molecules": see ref. 8) which directly encode most of the general facts which are needed.

### Special versus general

Now, what is wrong with having 'real-world' knowledge embodied directly in lower-level code in this way? If the program works satisfactorily, is that not enough? What more objective test could one have of a program's abilities? AI workers can be placed into two camps by the answers they give to such questions. We could call these the specialists and the generalists.

The former would tend to say there was nothing wrong with such encoding, that a program should be judged by its behaviour. They have traditionally been concerned to get programs to perform well in limited task areas, using whatever means come to hand. Joel Moses, author of a very successful program for symbolic integration, makes spirited defence

of this point of view<sup>9</sup>. Generalists (of whom I am one), would argue, on the other hand, that having general schemes for representing knowledge is an essential step in capturing the adaptability of intelligent behaviour. Purely on engineering grounds, it is very hard to alter information encoded at too low a level: SIR, for example, had to be almost entirely rewritten in order to extend its repertoire of conceptual relationships by just one addition; and was indeed abandoned for precisely that reason. Kelley makes similar remarks about his head-finding program.

More profoundly, since learning would seem to consist largely, in the addition to, or alteration of, a program's knowledge-store, it is vital that this knowledge be represented in a form in which it can be easily altered and added to, if meaningful learning by programs is to take place. Winston's concept-learning program, for example<sup>10</sup>, which can be taught descriptions of simple assemblies of bricks (arches, towers, piles), by showing it examples and counterexamples, depends for its abilities on having a very clean representation of concepts (in a network, in fact, although that is not essential) which can be progressively altered by local increments.

More fundamentally still, it seems to me that we should query whether knowledge encoded at such a low level is really present in the program at all. That a certain mechanism succeeds in a task is not adequate evidence alone that it in any sense knows what it is doing. A cabbage leaf channels rain to the root of the plant very efficiently: but we would not say that the cabbage knew about rain.

For several years in the mid-1960s, the 'generalist' and 'specialist' themes in AI research developed almost independently. Some people worked on both sides of the methodological gulf at once: but even then, there seemed to be very little generality visible in the *ad-hoc* specialist performance programs; and even those of the generalist persuasion were forced to the use of *ad-hoc* coding in order to get adequate efficiency in actual working systems. Many attempts were made to combine a general scheme for representing knowledge with a general-purpose heuristic mechanism controlling the application of the processing rules. But these always resulted in systems which, when applied to realistically difficult tasks, got lost in uncontrollably large search spaces. This is the now famous 'combinatorial explosion'. For several years, this uncomfortable situation was blamed on the 'weakness' of the general-purpose problem solvers. It was generally considered that when these became more 'powerful', as a result of identifying ingenious general heuristic mechanisms, the situation would improve. Great hopes were attached especially to the development of logical theorem-proving programs.

### Internal and external information

As is now, in retrospect, clear, this diagnosis was mistaken. The fault lies not in the general heuristic mechanisms, but in the interface between those mechanisms and the knowledge represented in the formalisms. The latter, consisting solely of information about the external task environment, gave no direct guidance to the mechanisms which processed the information. And without such guidance, no mechanism could possibly be 'powerful' enough to make appropriate inferences.

Consider, for example all the facts which an adult knows about people. One of them is probably that people bleed if you cut them; another, that they can walk. If you want to get somebody across a room, the second might be relevant, the first not: but that fact is not about the world, but about how to think about the world.

The point is similar to the platitude with which we began: the magic mechanism must be replaced by appropriate knowledge. But in this case, information is required not about the external task environment, but rather about the internal 'environment' of the scheme's processing rules. In a word, the system must represent, and use, information about its own thought processes.

And as we have seen, a description of a process can be

regarded as a program. It is not surprising, therefore, that the first expressions of this insight took the form of advocating the use of a suitable programming language as a way of representing knowledge of the external environment. This is the content of the term 'procedural representation of knowledge'<sup>11-13</sup>.

It is important to emphasise at this point that we have not simply moved full circle, back to the specialist programs with their 'knowledge' rigidly encoded in conventional programs. Rather, the proposal was to lift some programming ideas to the same level as that of the general representational schemes, in the hope of retaining much of the plasticity and clarity of the schemes already in use, but allowing the expression of information which controls the inferential activity. These programming languages are very high level.

The first programming language of this kind to be developed was MICROPLANNER<sup>14</sup>, and a brief example will show how such control information can be encoded in it. Consider the statement: if a black cat crosses your path, you will be lucky. Expressed in a logical notation, this would be something like: ((Exists X) Black(X) and Cat(X) and Crosses(X,path(you))) = > Lucky(you).

In MICROPLANNER, it can be expressed in many ways, depending on what you want to do with it. For example:

```
(CONSE (LUCKY? YOU)?)
  (PROG(X) (GOAL (BLACK?X))
    (GOAL (CAT?X))
    (GOAL (CROSSES?X PATH?YOU)))
```

The brackets are an inheritance from LISP, but what this means is, if you want to prove that YOU are lucky, try proving that there is a thing which is black and a cat and crosses your path—in that order. Now, in fact, this is probably a very inefficient way to establish this, as there are a lot more black things than things which cross your path. A better version might be:

```
(PROG (X) (GOAL (CROSSES? X PATH? YOU))
  (GOAL (CAT?X))
  (GOAL (BLACK?X)))
```

More dramatically, it can also be expressed

```
(ANTE (CROSSES?X PATH?YOU)
  (IF (AND (GOAL (BLACK?X))
    (GOAL (CAT?X))
    (ASSERT (LUCKY? YOU))))
```

which means, whenever it is asserted that anything crosses your path, see if it is a black cat: if so, assert that you are lucky. This is quite a different way of using the same fact. Notice, though that the level of these programs is similar to that of the logic: the MICROPLANNER system can clearly be seen to be performing inferences. MICROPLANNER is as different from LISP, as LISP is from machine code.

Facilities of this sort were first used, to powerful effect, in Winograd's now well known program<sup>11</sup> which held an English conversation with a human user. The demonstration was very dramatic, and since then this and other, similarly high level, programming languages have come into widespread use in AI work.

A high level programming language is based, we have said, on a conceptual device whose activity it describes. Now, MICROPLANNER's device is not much like a machine at all: it is, rather, an idealised 'thinker'. States of the device contain structured collections of assertions—the thinker's beliefs—and rules for manipulating them. Transitions between states correspond to inferences. Winograd's program used this directly to model its beliefs. Successors to MICROPLANNER retain these essential outlines, usually with more highly structured states. This gives a new view of computation as controlled inference, and of machines as essentially devices for storing assertions<sup>15</sup>.

This differs radically from the conventional semantics of programming languages. It is too soon to tell how far-reaching an effect it will have on our view of computing in general, but it seems to be unlikely to be negligible. Hewitt, who designed MICROPLANNER, has developed, for example, a very general theory of computing based on the idea of an 'actor'<sup>16</sup>.

The now widespread conception of a programming language



as a representation of knowledge explains the interest in programming as an activity, with which we began. For, now, programming is expressing knowledge; debugging is learning; and reasoning about programs is thinking about knowledge—one could say, introspection. Sussman's work<sup>10</sup> on automatic debugging of programs is a good example: his system analyses, classifies and corrects various kinds of programming error in simplified MICROPLANNER programs, such as the achievement of one goal being undone by a later action (this is a 'protection violation bug'). He regards this debugging as a model of a theory-formation process: start with a simple theory and enrich it by looking closely at the places it does not work. The program is the theory.

There is by no means universal agreement on the best way of expressing process-control information. It is no longer fashionable, indeed, to argue that a programming-language notation is essentially the best way to convey this information. I am myself working on a more direct notation for expressing such knowledge. But what is accepted now, by almost all AI workers, is that much of the information stored in an AI program has to be about what to think, as well as about what is true. This distinction cuts across the arguments about deductive or analogical assertions mentioned earlier: and it represents, I believe, a real advance in our understanding of thinking.

In AI, a program might be said to be aware of a thing when it has a sufficient amount of knowledge of that thing explicitly

represented and available for making inferences. In these terms, the 'procedural representation of knowledge' is a step towards the construction of programs which are aware of their own thought processes. One could think of a worse definition of consciousness.

<sup>1</sup> *Nature*, 250, 533–534 (1974).

<sup>2</sup> Higman, B. A., *A comparative study of programming languages* (Elsevier, Amsterdam, 1967).

<sup>3</sup> Moore, J., and Newell, A., *Knowledge and Cognition* (edit. by Gregg, L.), (Lawrence Erlbaum Associates, Potomac, 1973).

<sup>4</sup> Kelley, M. D., *Machine Intelligence*, 6, 397–409 (Edinburgh University Press, Edinburgh, 1971).

<sup>5</sup> Raphael, B., in *Proc. Fall Joint Computer Conference* (Association for Computing Machinery, New York, 1964).

<sup>6</sup> Schank, R., et al., in *Proc. Third int. joint Conf. Artificial Intelligence*, 255–261 (Stanford University, 1973).

<sup>7</sup> Wilks, Y., *Nature*, 252, 275–278 (1974).

<sup>8</sup> Reiger, C., thesis, Stanford Univ. (1973).

<sup>9</sup> Moses, J., Technical report MAC-TR-47 (Project MAC, Massachusetts Institute of Technology, 1967).

<sup>10</sup> Winston, P. H., *Report 231* (Artificial Intelligence Laboratory, Massachusetts Institute of Technology, 1970).

<sup>11</sup> Winograd, T., *Understanding Natural Language* (Edinburgh University Press, Edinburgh, 1972).

<sup>12</sup> Hewitt, C., *Report 258* (Artificial Intelligence Laboratory, Massachusetts Institute of Technology, 1972).

<sup>13</sup> Minsky, M., and Papert, S., *Progress Report* (Artificial Intelligence Laboratory, Massachusetts Institute of Technology, 1973).

<sup>14</sup> Sussman, G., Winograd, T., and Charniak, E., *Micro-planner Reference Manual* (Artificial Intelligence Laboratory, Massachusetts Institute of Technology, 1973).

<sup>15</sup> Hayes, P. J., in *Proc. Second math. Found. computer Sci. Symp.* (Czech. Academy of Sciences, Prague, 1973).

<sup>16</sup> Hewitt, C., *Proc. Third int. Joint Conf. Artificial Intelligence*, 235–245 (Stanford University, 1973).

<sup>17</sup> Sussman, G., *Report 297* (Artificial Intelligence Laboratory, Massachusetts Institute of Technology, 1974).

## articles

# Tectonic implications of Precambrian shear belts in western Greenland

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*The occurrence of four Precambrian shear zones in western Greenland may point to the existence of a lithospheric plate during Proterozoic times. The large scale, ductile, transcurrent movements can be interpreted as tectonically deeper expressions of major transcurrent faults and may represent the internal distortion of a Precambrian continental block.*

WHETHER the plate tectonic regime which characterised at least the later part of the Phanerozoic was of equal significance during Precambrian times is one of the outstanding geological problems of the present time. One approach to this problem is to examine the nature and distribution of large scale Precambrian displacements, to establish whether they are compatible with the neotectonic plate model (or with a modified version of this model, such as that requiring extensive internal distortion of lithospheric plates<sup>1</sup>), or whether they represent an entirely different tectonic system. The occurrence of four major shear belts, up to 40 km wide, in the Precambrian of western Greenland (Fig. 1) suggests that intracontinental displacements were significant in late Archaean–early Proterozoic times. The shear belts represent zones of mainly ductile, simple shear strain with dominantly transcurrent displacements of possibly

up to 200 km. At higher tectonic levels and at the surface these shear belts would have been expressed as major transcurrent faults. The characteristics of ductile zones of simple shear have already been described<sup>2–4</sup>.

## Nordre Strømfjord Shear Belt

This belt is situated in the central part of the Nagssugtoqidian mobile belt (Fig. 1) and can be traced for about 150 km between the coast and the icecap. The coastal half of the belt, which is the only part so far investigated in detail<sup>5</sup>, consists of strongly deformed granulite facies gneisses for which a shear strain ( $\gamma$ ) of 6 has been calculated. The belt is 15 km wide in the coastal section and so this shear strain implies a displacement of at least 100 km across the belt, including the transitional zones on either side. The shear strain has been calculated from the reorientation of earlier planar structures, the rotation of which shows the displacement to have been subhorizontal and sinistral, with a subvertical shear plane. The orientation of planar structures across the belt varies in a manner consistent with a wedge-shaped profile for the belt, narrowing upwards, with a wedge angle close to 40°.

Towards the north-east the shear belt is crossed by an amphibolite facies–granulite facies boundary which postdates the shear deformation. The decrease in metamorphic grade

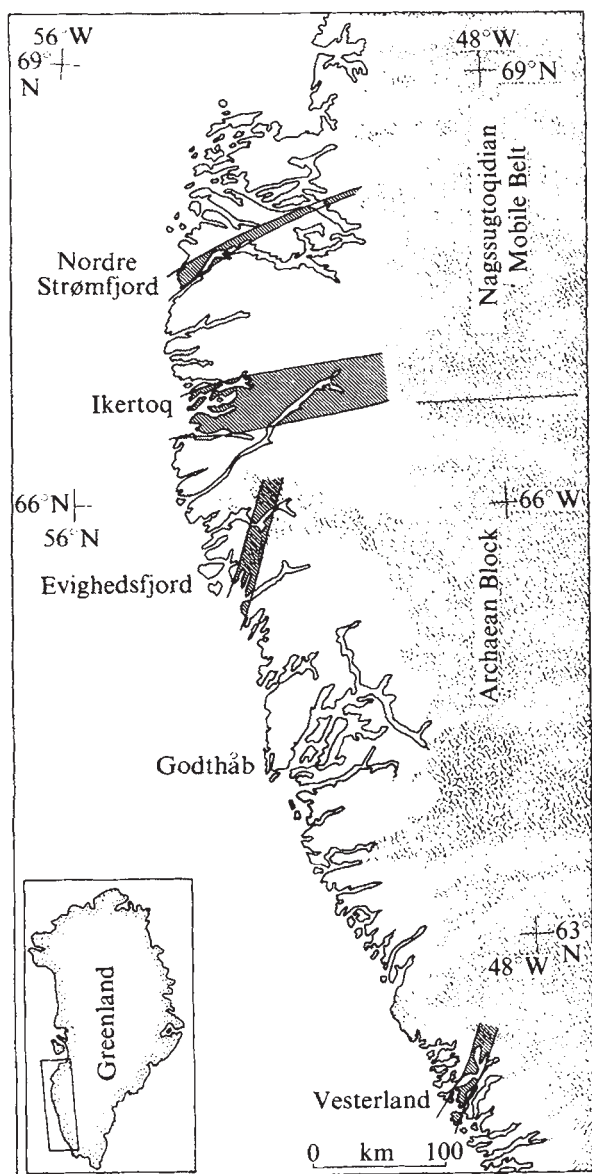


Fig. 1 Major shear belts in the central area of western Greenland.

from the coast towards the icecap is accompanied by a decrease in width of the shear belt from 15 km to approximately 7 km, which is compensated for by an increase in shear strain, so that displacement across the belt remains almost constant regardless of width. The eastward decrease in both width and metamorphic grade can be interpreted as the result of the differential uplift, by about 10 km, of the coastal region relative to the inland region (Fig. 2). Numerous occurrences of pseudotachylite have been recorded in the Nordre Strømfjord region<sup>6,7</sup>, but not within the shear belt.

### Ikertøq Shear Belt

This belt is 40 km wide, striking ENE with an exposed length of 150 km, and consists of highly deformed amphibolite facies gneisses bounded on both the northern and southern sides by granulite facies gneisses unaffected or only slightly affected by the shear movements<sup>8,9</sup>. An elongate block of granulite facies gneiss with maximum width of 12 km occurs within the shear belt and is characterised by weak or absent shear deformation; we regard this block as a large augen structure. A simplified map of the facies distribution in the coastal region is shown in Fig. 3. The structural history of the shear belt is complicated in many places by later ductile overthrusting, but where these later effects are absent, as in Itivdleq Fjord, the original

characteristics of the shear belt are preserved. In Itivdleq the intense L-S tectonite fabrics are consistent with a sub-vertical shear plane, with low angle stretching directions ( $x$  axis) corresponding to dextral transcurrent movements. Estimates of the shear strain indicate values greater than  $\gamma = 6$  which, if extrapolated to the full width of the Ikertøq Shear Belt, would correspond to a minimum displacement of over 150 km when allowance is made for the Kingaq and other smaller augen.

The granulite facies rocks bounding the shear belt, and within the Kingaq augen, are structurally distinct from the amphibolite facies rocks of the shear belt in as much as tectonite fabrics related to the shear movements are either weak or absent, and large scale, open, fold interference patterns are present. Discrete ductile shear zones, up to 50 m wide do occur within the granulite facies areas, however, and are characterised by retrogressive amphibolite facies assemblages. These small shear zones are either subparallel or oblique to the regional shear direction, and have variable movement directions.

Brittle movement zones, with pseudotachylite and pseudotachylite breccias, occur throughout the Ikertøq Shear Belt, but are most abundant within 5 km of both the northern and southern boundaries. The strike directions of the zones of brittle movement are usually parallel to those of the gneiss fabrics although the directions of movement are mostly unknown. The brittle movements extended over a long period of time; the earliest occurrences alternated with ductile movements while the rocks were still at metamorphic temperatures. The latest pseudotachylites postdate dykes of Cambrian age.

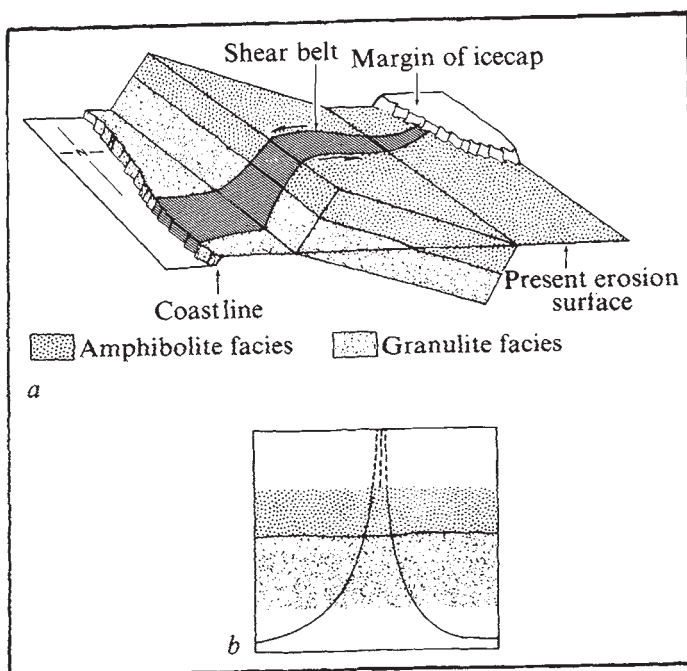
An important difference between the Ikertøq and Nordre Strømfjord shear belts is that in the Ikertøq Belt the mineral facies boundaries are very nearly coincident with deformation boundaries, whereas the mineral assemblage of the Nordre Strømfjord belt postdates the deformation, and metamorphic and deformation boundaries are apparently unrelated to one another.

### Evighedsfjord Shear Belt

This belt<sup>10</sup> has not yet been investigated in detail and its south-westerly continuation (Fig. 1) is conjectural.

In Evighedsfjord it is expressed as steep zones of highly

Fig. 2 Nordre Strømfjord Shear Belt. Interpretation as an oblique section brought about by differential uplift (tilt exaggerated); b, extrapolation of vertical section through the shear belt, showing suggested upward transition to fault zone and downward widening into horizontal boundary.





deformed, amphibolite facies gneisses retrogressively metamorphosed from surrounding, undeformed, granulite facies rocks. The western margin is about 10 km wide: granulite and amphibolite facies alternate in linear strips on a scale of tens or hundreds of metres. The amount and direction of displacement have not been established, but smaller shear zones with similar trend and attitude are the result of sinistral transcurrent movement. Pseudotachylite veins and breccias are abundant, and are concordant with the steep fabric of the highly deformed amphibolite facies rocks, although individual veins are transgressive. Although they occur mostly in the amphibolite facies gneisses, there are some pseudotachylite breccias in granulite facies rocks otherwise unaffected by the shear movements. The pseudotachylites postdate the ductile deformation but all of those seen predate the Kangâmiut dyke swarm<sup>10</sup>, the emplacement of which can be shown to closely follow the ductile deformation.

### Vesterland Shear Belt

Although the Frederikshåb district in which this shear belt occurs has been systematically mapped on a scale of 1:20000 (ref. 11) the shear belt is not known in detail as it was not recognised as such until the later stages of the mapping programme. The belt has a NNE trend and can be traced over a distance of 60 km from Vesterland on the coast<sup>12</sup> to the icecap in amphibolite facies gneiss terrain<sup>13</sup>. It is about 10 km wide and the marked tectonite fabric—and its orientation—are consistent with a subhorizontal shear direction. Two domains of extremely high shear-strain occur at the margins of the belt. Detailed analysis of the northern part of the shear<sup>14</sup> belt has shown the strain to be very variable, from  $\gamma < 1$  to  $\gamma \geq 6$ , and, consequently, it has not yet been possible to integrate the strain across the belt to determine the amount of displacement, although it is clearly dextral.

It is likely that other shear belts will be recognised in western Greenland in the future. The possibility that a major shear belt exists in Godthåbsfjord has been suggested<sup>14</sup>.

### Age and seismicity of the shear belts

The limited radiometric data on rocks within and bordering the shear belts, and the ages of displacements relative to dyke swarms, show that movement on all four shear belts is likely to have been initiated 2,000–3,000 Myr ago, although there is no reason to suppose that all were initiated at the same time. Movement along the northern margin of the Ikertôq Belt occurred as recently as the Mesozoic<sup>15</sup>, and faulting of Jurassic dykes along NNE fractures close to, and within, the Vesterland Belt has been recognised<sup>16,17</sup>.

The abundant occurrence of pseudotachylite in two of the shear belts, and its presence in a third, is a result of brittle deformation closely associated with the ductile shear deformation, although pseudotachylite formation also occurred during later movements. The change from ductile to brittle behaviour and *vice versa* at this level in the crust is regarded primarily as a result of variation in strain rate rather than of short term changes in temperature or tectonic level. We believe the formation of pseudotachylite by shear fracturing to be necessarily accompanied by the release of elastic strain sufficient to generate seismic waves (see refs 18 and 19), although the generation of seismic waves is not necessarily accompanied by pseudotachylite formation.

This conclusion implies that two and possibly three of the shear belts described represented well defined linear seismic zones at the time of their formation, at the tectonic level now exposed. At higher tectonic levels all four belts were likely to have been seismically active. The depth represented by the tectonic level exposed at present is uncertain, but is thought to be 10–15 km below the contemporary surface; that is, at a depth similar to the deeper earthquake foci on the San Andreas<sup>20</sup> and Rhine Graben<sup>21</sup> systems at the present time. We anticipate that the deeper, aseismic displacements on the San Andreas,

Rhine and similar fractures are represented by ductile shear zones comparable to the examples in Greenland. The comparison with neotectonic, linear seismic belts can be taken a stage further in the case of the Nordre Strømfjord Shear Belt, the truly linear nature of which is indicated by its curving map outcrop, which is consistent with a small circle configuration on a sphere. (We emphasise, however, that the geometrical identification of a small circle configuration does not necessarily imply any dynamic significance.)

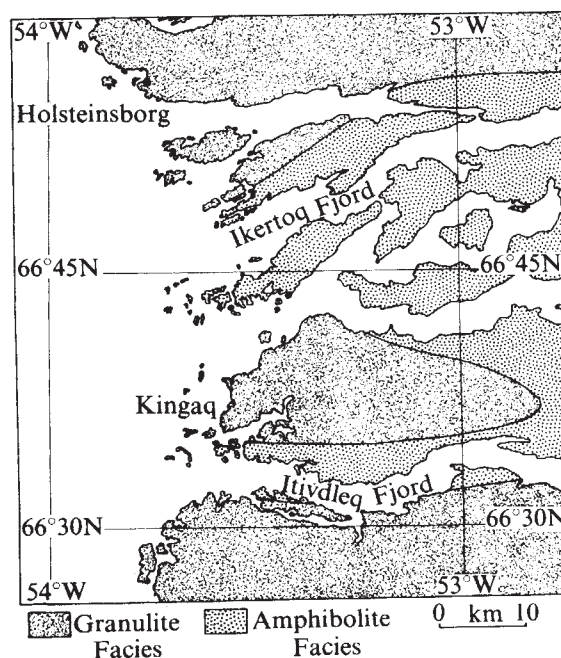
### Implications for Proterozoic tectonics

The shear belts represent singular events which cannot be related to one another by the traditional orogenic concept. A suggestion<sup>22</sup> that the Nagssugtoqidian boundary, with which the Ikertôq Belt is associated, may represent a plate collision boundary has not received subsequent support, although the evidence of ductile overthrusting on which it was based remains valid.

It is difficult to envisage large scale transcurrent displacements of the type described without involving the existence of a horizontal layer along which the horizontal translation could be accommodated; the existence of such a decoupling zone defining the base of the lithosphere is, however, the *sine qua non* of the plate tectonic theory, although the evidence for the existence of such a layer derives almost entirely from investigation of activity at plate edges. We suggest, therefore, that the continental mass within which the Precambrian shear belts were formed has the essential characteristic of a lithospheric plate, that is, a lower bounding surface which permitted differential translation relative to the underlying material. The downward widening of the Nordre Strømfjord Shear Belt (Fig. 2) if continued, may show how vertical and horizontal shear layers are connected.

Discussion of the significance of the shear belts in terms of plate rigidity and plate margins is circumscribed by problems of definition. Neotectonic plate boundaries are represented by discontinuities; although the Precambrian shear belts do not represent significant discontinuities at the tectonic level now exposed they would have done so at higher tectonic levels. On one scale the shear belts represent non-rigid zones bounding more rigid crustal blocks, but on a larger scale they can be thought of as representing distortion within a larger continental mass. The question of whether the shear belts could be

Fig. 3 Metamorphic facies distribution in the coastal section of the Ikertôq Shear Belt.



regarded as plate boundaries cannot, therefore, be answered simply. There is no doubt, however, that they represent displacements within a single continental mass and alternative conclusions can be based on this premise.

Rigidity of lithospheric plates is only relative and the internal rigidity of neotectonic plates may have been overemphasised, although the evidence for the lack of overall distortion of shape<sup>23</sup> is very persuasive. The Precambrian shear belts may be expressions of occurrences that are qualitatively comparable to the intraplate activity now represented by, for example, the East African Rift system, the Rhine Graben, the North Sea Graben, or the South Siberian Fault system. The apparently more intense Precambrian intraplate activity could be explained in terms of the greater time period represented by the western Greenland shear belts, which is possibly longer than the Phanerozoic.

Alternatively, if the shear belts represent significantly greater intraplate activity during the Precambrian (thus indicating a significantly less rigid lithosphere) this would be consistent with a relative mechanical weakness resulting from a greater width–thickness ratio of the Precambrian lithosphere. This could be because either the lithosphere was thinner, or because there was a large Precambrian plate, or it could be caused by a combination of the two.

If, as is often assumed, middle and early Precambrian times were characterised by higher heat flows, a corresponding reduction in the thickness of lithospheric plates would be expected insofar as the lithosphere–asthenosphere boundary is defined by an isotherm. The consequent reduction in mechanical strength, and its effect on tectonic behaviour, could then be compared with the more fractured nature of neotectonic plates where these are thinnest as a result of higher heat flows adjacent to constructive boundaries<sup>23</sup>. Variation of rock composition, and melting point, with depth is, however, likely

to complicate any direct relationship between thermal gradient and lithospheric thickness. The palaeomagnetic evidence<sup>24</sup> for the existence of a Proterozoic supercontinent at least 15,500 km wide, and possibly 21,000 km wide, is sufficiently convincing for the size of the Precambrian plate to be regarded as an important factor when mechanical behaviour is considered. If both thickness and size varied then the width–thickness ratio of the Precambrian plate would, perhaps, be 3–5 times greater than that of a typical neotectonic plate.

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- <sup>1</sup> Sutton, J., and Watson, J. V., *Nature*, **247**, 433 (1974).
- <sup>2</sup> Ramsay, J. G., and Graham, R. H., *Can. J. Earth Sci.*, **7**, 786 (1970).
- <sup>3</sup> Escher, A., and Watterson, J., *Tectonophysics*, **22**, 223 (1971).
- <sup>4</sup> Escher, A., Escher, J., and Watterson, J., *Can. J. Earth Sci.* (in the press).
- <sup>5</sup> Bak, J., Korstgård, J. A., and Sørensen, K., *Tectonophysics* (in the press).
- <sup>6</sup> Jensen, V., *Rep. geol. Surv. Greenl.*, **15**, 22 (1968).
- <sup>7</sup> Jensen, V., *Nature*, **233**, 188 (1971).
- <sup>8</sup> Bridgwater, D., Escher, A., Nash, D. F., and Watterson, J., *Rep. geol. Surv. Greenl.*, **55**, 22 (1973).
- <sup>9</sup> Watterson, J., *ibid.*, **65**, 33 (1974).
- <sup>10</sup> Escher, A., Escher, J., and Watterson, J., *ibid.*, **28**, 21 (1970).
- <sup>11</sup> Jensen, S. B., *ibid.*, **19**, 33 (1969).
- <sup>12</sup> Watterson, J., *Medd. Grönland*, **175**(6), (1968).
- <sup>13</sup> Bak, J., thesis, Aarhus University (1974).
- <sup>14</sup> McGregor, V. R., and Bridgwater, D., *Rep. geol. Surv. Greenl.*, **55**, 29 (1973).
- <sup>15</sup> Watterson, J., *Nature*, **253**, 520 (1975).
- <sup>16</sup> Walton, B. J., and Arnold, A. R., *Medd. Grönland*, **190**(5), (1970).
- <sup>17</sup> Rivalenti, G., and Rossi, A., *ibid.*, **192**(5), (1970).
- <sup>18</sup> Francis, P. W., and Sibson, R. H., *The Early Precambrian of Scotland and related rocks of Greenland* (edit. by Park, R. G., and Tarney, J.), **95** (1973).
- <sup>19</sup> Sibson, R. H., *Nature*, **243**, 66 (1973).
- <sup>20</sup> Eaton, J. P., Lee, W. H. K., and Pakiser, L. C., *Tectonophysics*, **9**, 259 (1970).
- <sup>21</sup> Illies, J. H., *Graben Problems* (edit. by Illies, J. H., and Mueller, S.) **4** (1970).
- <sup>22</sup> Bridgwater, D., Escher, A., and Watterson, J., *Phil. Trans. R. Soc.*, **A273**, 513 (1973).
- <sup>23</sup> Le Pichon, X., Francheteau, J., and Bonnin, J., *Plate Tectonics* (Elsevier, Amsterdam, 1973).
- <sup>24</sup> Piper, J. D. A., *Nature*, **251**, 381 (1974).

# Groenlandaspis in Antarctica, Australia and Europe

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*Groenlandaspis is a member of the Arthrodira, a group of Devonian armoured fishes. In the past Groenlandaspis has been found only in eastern Greenland but now Alexander Ritchie has recognised at least six species from sites in Greenland, Europe, Australia and Antarctica. His account demonstrates that these arthrodiras enjoyed a very wide geographical distribution during the Upper Devonian and that the family can be traced back into the Middle and Lower Devonian.*

*Groenlandaspis mirabilis* Heintz, from the late Upper Devonian or earliest Carboniferous of eastern Greenland, is one of the last known members of the armoured arthrodiran fishes. The original fossil material<sup>1–3</sup> is limited in quantity, disarticulated and fragmentary and there is insufficient to justify either a full description of the species or a reconstruction. The specimens were collected mostly by Scandinavian expeditions to eastern Greenland in the 1920s and 1930s. Although very poorly known, *Groenlandaspis* is generally accepted as a very late representative of a very important group of arthrodiras (the arctolepids or dolichothoracids) which reached their peak in numbers and diversity much earlier in Lower and Middle Devonian times. Until very recently *Groenlandaspis* had not been positively recorded from outside Greenland.

As a result of field discoveries in Australia and Antarctica between 1968 and 1974, and finds made in existing European museum collections in early 1973, knowledge of the genus and its closest relatives has increased dramatically. The family Groenlandaspididae is now known to have occurred on at least four continents and to range in time from Lower Devonian until the very end of the period and perhaps even into the Carboniferous. The genus can now be recognised from material discovered in southern Victoria Land, Antarctica, half a world away from its original occurrence in Greenland. At the time of writing at least six species of *Groenlandaspis*—several of them new to science—are known from the Upper Devonian of eastern Greenland, Ireland, England, south-eastern Australia, and Antarctica. A new genus of the family Groenlandaspididae has been recovered from the Middle Devonian of western New South Wales, Australia and the family can be traced back into the Lower Devonian in the form of *Tiaraspis*, a small arthrodire from Germany<sup>4</sup>.

Most of these occurrences are now represented by abundant, well-preserved material. The discovery of *Groenlandaspis* on four widely scattered continents indicates a virtual worldwide distribution in the Upper Devonian and the genus may well prove of considerable value in intra and intercontinental correlation, supplementing the evidence already available from the antiarchs, *Bothriolepis* and *Remigolepis*, with which it is commonly, though not invariably associated.



## Geographical distribution

The sequence of events which led to the discovery and recognition of *Groenlandaspis* on four continents has been described elsewhere<sup>5</sup>. Devonian fish remains from Antarctica were first discovered by members of Scott's last expedition in 1911–12 and were later described by Woodward<sup>6</sup>. Material from other sites in southern Victoria Land, collected by Gunn and Warren<sup>7</sup> during the 1957–59 Commonwealth Trans-Antarctic Expedition, has been described by White<sup>8</sup>. The very fragmentary material, apparently of Upper Devonian age, included arthrodires, antiarchs, palaeoniscids, crossopterygians and elasmobranchs.

Two recent geological expeditions to the same area recovered more, and better preserved, Devonian fish material from many scattered localities<sup>9,10</sup>. Preparation of this material has revealed that most of the remains of arthrodires from the Aztec Siltstone of southern Victoria Land can be attributed to one or more species of *Groenlandaspis*.

By a curious coincidence the same genus came to light in Australia while the Antarctic studies were in progress. In May 1972 I investigated two Upper Devonian sites, 40 miles apart, in central New South Wales (NSW) and I recovered material representing two new and quite distinct species of *Groenlandaspis* from each locality. The only previous record<sup>11</sup> of *Groenlandaspis* in Australia is probably incorrect although a member of the Groenlandaspididae is now known to occur in the same fauna, that of the Mulga Downs Group of Middle Devonian age.

The new Upper Devonian records, both from the Hervey Group, come from the Cloghnan Shale in the Jemalong Range, some 20 miles west of Forbes, NSW and the Hunter Siltstone, Redcliff Mountain, 11 miles NNE of Grenfell, NSW. Large quantities of fish-bearing material from these sites are under preparation and study in the Australian Museum. In both cases *Groenlandaspis* is associated with *Bothriolepis* and *Remigolepis* spp. but *Phyllolepis* is present at Jemalong and absent at Grenfell and the faunas are quite distinct in other respects.

Between December 1972 and April 1973 I carried out a study tour of Devonian vertebrate collections in Europe and discovered two more species of *Groenlandaspis* in museum collections, the first records of the genus from Europe. Both came from very late Upper Devonian deposits and from faunas which had been known as early as the 1850s.

The first came from the Portishead Beds, exposed on the shores of the River Severn 8 miles west of Bristol in south-western England. Small arthrodiran plates occur, tentatively

identified<sup>12</sup> as *Coccosteus? disjectus* Woodward, a species recorded from the almost contemporaneous late Upper Devonian Kiltorcan Beds in southern Ireland. These small plates, from a collection housed in the Department of Geology, University of Bristol, possess, however, the diagnostic feature of a species of *Groenlandaspis*.

The second European discovery of *Groenlandaspis* came to light in the Sedgwick Museum, University of Cambridge. A small collection of unidentified, unlocalised and unregistered fish plates again showed the distinctive and by now familiar features of *Groenlandaspis* but of a species quite distinct from the Bristol occurrence. I also examined a small collection of fish plates in the Royal Scottish Museum, Edinburgh, which proved to be from the same source as the material from the Sedgwick Museum. Both had apparently come from a late Upper Devonian horizon near Kiltorcan in southern Ireland, a site better known for its abundant and well preserved plant fossils than for the associated fish fauna. The material came originally from the Geological Survey in Dublin, having been excavated in the 1850s and 1860s. During a brief visit to Dublin I established, from the original registers, that over 400 fish plates had been recovered from the Kiltorcan Beds between 1858 and 1861 (ref. 13) and that shortly afterwards representative samples of the fossil flora and fauna had been sent to Cork, Galway, Belfast, Dublin (Trinity College), Edinburgh, Oxford, Cambridge and London. Although some of this material has been lost over the years a surprising amount has now come to light as the result of recent enquiries.

Some of the Kiltorcan arthrodiran plates were described originally<sup>14</sup> by Woodward as *Coccosteus disjectus*, an identification generally accepted in stratigraphic accounts until recently<sup>15</sup>. Miles and Westoll<sup>16</sup> pointed out that *C. disjectus* was not a valid species of *Coccosteus* and that it may even have been an arcolepid and not a brachythoracid arthrodire. Their suspicions were well founded; virtually all of the Kiltorcan arthrodiran plates I studied belong to *Groenlandaspis* and can be placed in one species—*G. disjectus* (Woodward). There is evidence to suggest the presence of a second species in the Irish material.

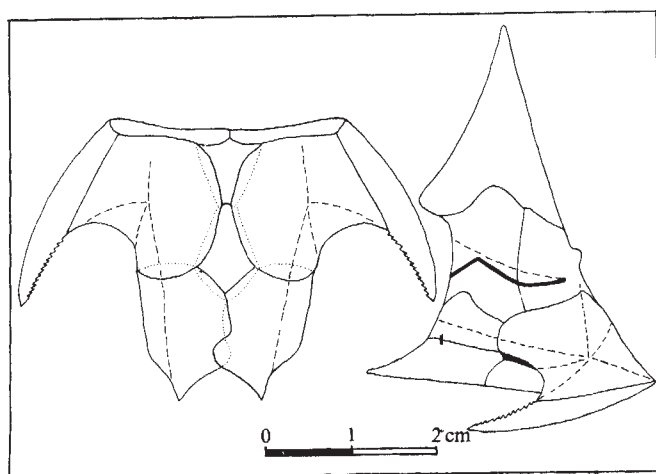
Re-examination of existing collections in the Australian Museum has led to the discovery of a third occurrence of *Groenlandaspis* in the Upper Devonian of NSW. A figured specimen among some fragmentary fish plates from the Hervey Range, north-west of Barks in NSW, which was formerly<sup>17</sup> identified tentatively as a "crossopterygian dermal plate" can now be recognised as a left anterior dorsolateral plate of *Groenlandaspis* sp.; three more plates of the genus have been discovered in the same collection, but there is insufficient material for specific identification. The original site has not yet been relocated.

## Middle and Lower Devonian Groenlandaspididae

All of the *Groenlandaspis* occurrences mentioned above are from undoubted Upper Devonian deposits. It is now apparent that *Tiaraspis* (Fig. 1), a small arthrodire from the Lower Devonian of Germany<sup>4</sup> is an early, possibly ancestral, member of the family. The apparent absence of Middle Devonian representatives of the family initially presented a problem but that has now been solved following the recognition of a new genus of the Groenlandaspididae in the Mulga Downs Group of western NSW which has yielded large quantities of dissociated arthrodiran material.

*Wuttagoonaspis*, a new arthrodiran genus<sup>18</sup>, forms the bulk of the fauna. But the abundant associated material contains many arthrodiran plates which cannot be assigned readily to a genus or species. It is now apparent that many of the plates display features approximately intermediate between those of *Tiaraspis* and *Groenlandaspis*. On other evidence the fish beds of the Mulga Downs Group are thought to be of early Middle Devonian age so the new member of the Groenlandaspididae present in the fauna, probably representing a new genus, falls neatly into place.

Fig. 1 *Tiaraspis subtilis* (Gross). Lower Devonian, Germany. Trunk armour seen in ventral and right lateral view. Overlap areas indicated by dotted line. (After Gross 1962.)



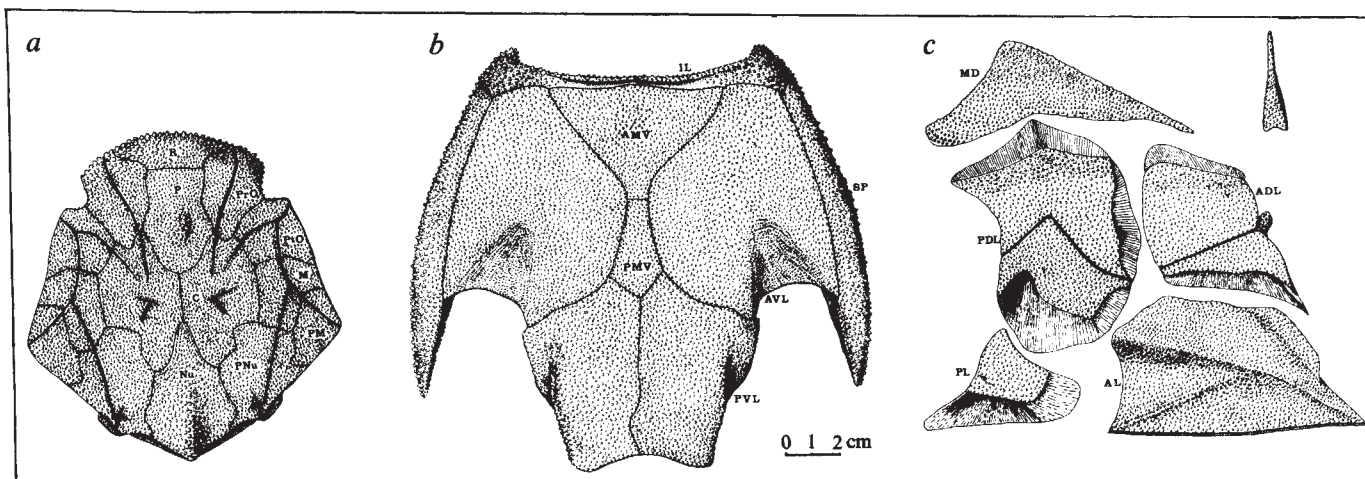


Fig. 2 *Groenlandaspis antarcticus* sp. nov., Upper Devonian, Aztec Siltstone, southern Victoria Land, Antarctica. a, Headshield; b, ventral trunk shield; c, dorsal and right lateral trunk plates, shown detached. All to same scale. ADL, PDL, anterior and posterior dorsolaterals; AL, PL, anterior and posterior lateral; AMV, PMV, anterior and posterior median ventrals; AVL, PVL, anterior and posterior ventrolaterals; C, central; IL, interlateral; M, PM, marginal and postmarginal; MD, median dorsal; Nu, nuchal; P, pineal; PNu, paranuchal; PrO, PtO, pre and postorbitals; R, rostral; SP, spinal.

## Relationship between *Groenlandaspis* and the Groenlandaspididae

Although it is generally agreed that *Groenlandaspis* belongs in the family Arctolepida Heintz 1937 (=Dolichothoraci Stensiö 1944) confusion exists concerning its relationships with other arctolepids, partly because there is inadequate material of the type species, *G. mirabilis* Heintz.

Denison<sup>19</sup> placed *Groenlandaspis* in the subfamily Phlyctaenaspinae whereas Obruchev<sup>20</sup> placed it in a family of its own—the Groenlandaspididae—and grouped it with the Holonematidae in a separate suborder, the Holonematoidae. Miles<sup>21,22</sup> has shown that the Holonematidae are brachythoracid arthrodires at an advanced coccosteomorph level of organisation, although they are phylogenetically distinct from all other brachythoracids. In spite of certain similarities between the Holonematidae and the Groenlandaspididae a close association seems unwarranted at present.

I propose a classification which is a modified version of that of Miles<sup>21</sup> in which the name Arctolepida (which has precedence) is preferred over the name Dolichothoraci, and in which the Tiaraspididae are merged with the Groenlandaspididae.

### Suborder Arctolepida

Family 1, Actinolepididae; 2, Phlyctaenaspidae; 3, Groenlandaspididae; 4, Williamsaspidae; 5, Aggeraspidae. Amongst these the Groenlandaspididae are most closely related to the Phlyctaenaspidae on the evidence from the trunk armour.

### Family Groenlandaspididae Obruchev 1964

Obruchev<sup>20</sup> and Romer<sup>23</sup> included two poorly known genera, *Grazosteus* and *Tropidosteus*, in the Groenlandaspididae but Miles<sup>22</sup> has shown that these forms are of uncertain affinities and they are not here retained in the family. At present only three genera are considered to be undoubted members of the Groenlandaspididae—*Groenlandaspis* (Upper Devonian); a new undescribed genus from Mulga Downs Group, western NSW (Middle Devonian); and *Tiaraspis* (Lower Devonian).

Groenlandaspididae: Euarthrodira with unreduced lateral trunk shield, long contact between lateral and ventral trunk shield. Pectoral fenestra of medium size. Spinal plate large. Median dorsal plate long, extremely narrow with high pointed laterally compressed dorsal ridge. Anterior and posterior dorsolateral plates high, short. Dorsolateral canal with angular, dorsally directed flexure over posterior dorsolateral plate. Paired anteroventrals absent. Craniothoracic articulation well developed, condyles situated rather close together. Headshield subpentagonal, posterior margin convex or angular.

### Groenlandaspis

Type species: *G. mirabilis* Heintz 1932. Groenlandaspis Series, Upper Devonian (Famennian)—? Lower Carboniferous (Tournasian). Eastern Greenland.

Discussion: *G. mirabilis* is a rather large arctolepid of which most of the plates of the trunk armour are known from isolated specimens and the headshield is known only from a natural mould of the posterior margin (and the articular fossae) and from an isolated central plate<sup>1-3</sup>. From our present complete knowledge of the genus, based on the Antarctic material, it is clear that most of the Greenland specimens are identified correctly, except that the central plate figured by Stensiö (ref. 2; No. 3, Fig. 12) is the left, not the right, and should be reversed. The posterior ventrolateral plate (PVL) figured by Miles (ref. 3; Fig. 9 A–D) at first sight seems to differ significantly from the PVL of *Groenlandaspis antarcticus* sp. nov. (Fig. 2b). During examination of the original *G. mirabilis* PVL in Copenhagen, it was found, however, that parts of two separate plates had been erroneously placed in close association, probably at the time of discovery in 1954. The posterior portion of the PVL, as restored, is incorrect and the shape almost certainly corresponded closely to that in *G. antarcticus*.

Although it would be difficult to distinguish between isolated or fragmentary plates of *Groenlandaspis antarcticus* sp. nov. and those of the type species, *G. mirabilis* Heintz, there are sufficient minor differences to warrant separation in distinct species, at least until more complete material of the Greenland form becomes available. The geographical separation alone suggest that it is unlikely that the Antarctic and Arctic faunas were conspecific.

The genus *Groenlandaspis* can now be described in detail for the first time based on the excellent material from Antarctica, Australia and Europe. Only the new Antarctic species is covered here but the diversity of the known species of the Groenlandaspididae is illustrated by a selection of the median dorsal plates of the new discoveries (Fig. 3 a–f); the incompletely known MD of the type species, *G. mirabilis* Heintz, is not shown here but seems to be close to that of *G. antarcticus* sp. nov. (Fig. 3a).

### *Groenlandaspis antarcticus* sp. nov. (Fig. 2 a–c).

Type: the Australian Museum F54334, almost complete headshield, from Mount Ritchie, western side of Deception Glacier, south Victoria Land, Antarctica.

Referred specimens: AM F54336, F54314 (partial headshields); F56348 (complete ventral shield); F54337 (MD); F54382 (ADL); F54343, F54379 (PDLs); F54389 (AL); F54406, F56349 (PLs); F56222 (AMV); F55669 (PMV); F54344 (PVL); F56321–2 (IL). Locality: all from same locality and horizon as type, Mount Ritchie, Unit 62, Section A4 (24), with exception of F54314 (Portal Mountain), F56348 (Mount Metchel, southern end), F55669 (Section A2, small nunatak, north of Alligator Peak).

Horizon: Upper Devonian, Aztec Siltstone, Taylor Group<sup>24</sup>.

Description and discussion: neither Woodward's material<sup>6</sup> nor that of White<sup>8</sup> from south Victoria Land contained specimens which can be positively attributed to *G. antarcticus* sp. nov. The incomplete headshield of an aberrant arthrodire *Antarctaspis momurdoensis* White from Mount Crean differs strongly from the cranial material of *Groenlandaspis* collected by VUWAE 15 in 1970–1. The trunk plates figured by White (ref. 8; Plate III, figs 1 and 2) as *Antarctolepis* show some groenlandaspid-like features but are quite distinct from the equivalent plates in *G. antarcticus*. There is some evidence for the presence of more than one species of *Groenlandaspis* in the Upper Devonian of Antarctica.

The cranial shield, poorly known in the type species from Greenland, can be restored fully from the Antarctic discoveries. An almost



complete specimen (AM F54334), recovered in many fragments from near the summit of Mount Ritchie, has been extracted and reconstructed. Most of the remaining material has also been prepared.

The restoration of the headshield of *G. antarcticus* (Fig. 2a) incorporates information from two other partial headshields and numerous fragments.

The nuchal plate (Nu) is not particularly large, six-sided, bluntly pointed posteriorly and sharply pointed anteriorly. The other median plates, the rostral and pineal (R, P), are separate, unlike in many arctolepids where they are apparently fused. The pineal plate (P) is unusually large, extending posteriorly almost to the centre of the shield, completely separating the preorbital plates (PrO) and partly separating the large centrals (C). The latter are rather irregular in shape for an arctolepid, reflecting the extensive development of overlap areas to a degree more commonly found in brachythoracid arthrodires. The articular fossae on the visceral surface of the paranuchal plates (PNU) are large, well developed and situated quite close together, as in *G. mirabilis*. The posterior margin of the PNU bears a prominent preglenoid process.

The cranial sensory canal pattern is fairly conventional except for one feature not often found in arctolepids—the supraorbital canal extends beyond the preorbital plate (PrO) on to the central plate (C).

The trunk armour is known in considerable detail from many specimens of isolated plates (Fig. 2c) and the ventral surface is restored (Fig. 2b) from an especially fine, virtually complete specimen recovered from the southern end of Mount Metchel, Skelton Névé.

The most distinctive plate of the *Groenlandaspis* trunk armour is undoubtedly the median dorsal (MD), an elongate, extremely narrow plate with a pointed dorsal crest and almost vertical lateral surfaces (Fig. 2c). The ventral margin is shallowly indented for the insertion of the equally distinctive high but short, anterior and posterior dorsolateral plates (ADL, PDL). A unique feature in this genus is that the dorsal portions of the right and left PDLs meet one another in the middle, inside the very narrow base of the MD. A broad rugose surface is developed over the area where they meet.

A characteristic feature of the PDL in *Groenlandaspis*, already known from *G. mirabilis* as shown by Stensiö (ref. 2; No. 3, Fig. 13), is the sharply flexed course of the dorsolateral canal crossing the plate. The anterior and posterior portions of the canal meet almost at right angles. This feature runs right through the family although it is not so extremely developed in *Tiaraspis* (Fig. 1).

The anterior lateral plate (AL) is large and, for an arctolepid, relatively low and long. The pectoral fenestra is closed behind by a smallish posterior lateral plate (PL) which is inserted dorsally into a deep groove in the PDL and attached ventrally to the dorsal lamina of the large posterior ventrolateral plate (PVL). On the ventral surface (Fig. 2b) the most significant character is the absence of paired anteroventral plates which are present in the Actinolepididae. The area is occupied by a large, semicircular anterior median ventral plate (AMV) which immediately suggests a close relationship between the *Groenlandaspidae* and the *Phlyctaenaspidae*<sup>25</sup>.

## Evolutionary trends

Through the known history of the *Groenlandaspidae* there is a noticeable trend towards progressive reduction in the height of the dorsal spine and a corresponding elongation of the median dorsal plate (MD). In the Lower Devonian *Tiaraspis* (Fig. 1) the MD is short, very high and acutely pointed; in the new genus from the Middle Devonian Mulga Downs Group of western NSW (Fig. 3f) the MD is slightly longer than high and less acutely pointed. With the exception of the Grenfell form (Fig. 3e) all of the Upper Devonian species of *Groenlandaspis* have an MD which is much longer than high (Fig. 3a-d); in all of them (Fig. 3a-e) the highest point is posteriorly placed and directed posterodorsally.

A similar series can now be constructed for the other trunk plates, most usefully for the distinctive ADL and PDL plates, but there is often considerable variation within each fauna, the extent of which is still being analysed.

## Relative age of the known *Groenlandaspidae*

The three representatives of *Groenlandaspis* from the Northern Hemisphere (Greenland, Ireland and south-western England) are all known from the youngest Devonian rocks in their respective areas. In Greenland the genus may last into the Tournasian. The NSW specimens all occur in the Upper Devonian Hervey Group of central NSW (near Forbes, Grenfell and Parkes) but none of them come from the latest part of the Devonian sequence in their respective areas and it seems probable that they are somewhat older than the species from the Northern Hemisphere.

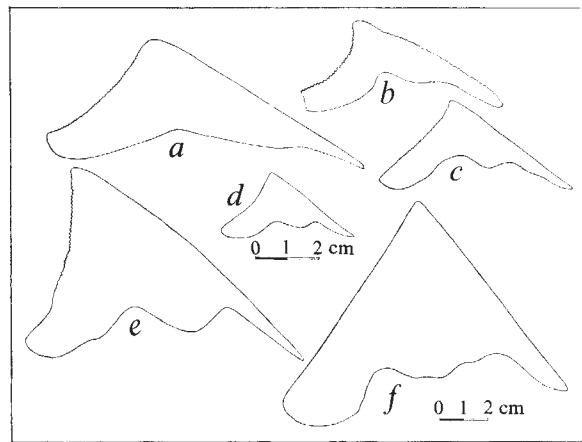


Fig. 3 Median dorsal plates of *Groenlandaspidae* in right lateral view. a-e, All Upper Devonian and to same scale; f, Middle Devonian, slightly reduced scale. a, *Groenlandaspis antarcticus* sp. nov., southern Victoria Land, Antarctica; from Australian Museum F54337; b, *G. disjunctus* (Woodward) Kiltorcan, Ireland; Institute of Geological Sciences, London Zo3897; c, *Groenlandaspis* sp. nov., Jemalong Range, Forbes, NSW, Australian Museum F56893; d, *Groenlandaspis* sp. nov., Portishead, near Bristol, England; Bristol University Geology Department, BUGD 9926, 17850; e, *Groenlandaspis* sp. nov., Grenfell, NSW, Australian Museum F56125; f, gen. et sp. nov. Mulga Downs Group, west of Cobar, NSW, Australian Museum F54567.

Accurate dating and correlation of the specimens from NSW must await analysis of the rich associated faunas, but one unexpected feature is that the closest resemblances are not between those species of *Groenlandaspis* which are geographically close. The Greenland and Antarctic species are alike in many respects as are the forms from Bristol, south-western England and Forbes, NSW. But the Irish *G. disjunctus* and the distinctive new species from near Grenfell, NSW could not be confused with any of the other known occurrences.

The value of the family for stratigraphic purposes has been demonstrated by the recognition of a *groenlandaspis* in association with a *Wuttagoonaspis* sp. in the northern Dulcie Range, about 130 miles north-east of Alice Springs, Northern Territory, Australia (G. Young, personal communication), a fauna which is clearly comparable to the Middle Devonian Mulga Downs Group of western NSW<sup>18</sup>.

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- <sup>1</sup> Heintz, A., *Skr. Svalbard Ishavet*, 42, 1–27 (1932).
- <sup>2</sup> Stensjö, E., *Meddr. Gronland*, 97, No. 1 (1934); No. 2 (1936); No. 3 (1939).
- <sup>3</sup> Miles, R. S., *Ark. Zool.*, 16 (2), 427–60 (1964).
- <sup>4</sup> Gross, W., *Palaont. Z.*, H. Schmidt-Festband, 45–63 (1962).
- <sup>5</sup> Ritchie, A., *Aust. Natur. Hist.*, 18, 28–35 (1974).
- <sup>6</sup> Woodward, A. S., *Br. Antarct. Terra Nova Exped.* 1910, 1, 51–62 (1921).
- <sup>7</sup> Gunn, B. M., and Warren, G., *Scient. Rep. Antarct. Exped.*, 10, 1–156 (1962).
- <sup>8</sup> White, E. I., *Scient. Rep., ibid. Antarct. Exped.*, 16, 1–26 (1968).
- <sup>9</sup> McKelvey, B. C., Webb, P. N., Gorton, M. P., and Kohn, B. P., in *The Geology of Antarctica* (edit. by Adie, R. J.), 345–352 (Universitetsforlaget, Oslo, 1972).
- <sup>10</sup> Ritchie, A., *Aust. Natur. Hist.*, 17, 65–71 (1971).

- <sup>11</sup> Rade, J., *J. Paleont.*, 38, 929–932 (1964).
- <sup>12</sup> Wallis, F. S., *Q. Jl Geol. Soc. Lond.*, 83, 760–786 (1928).
- <sup>13</sup> Bailly, W. H., Explanation to accompany Sheets 147 and 157 of the maps of the Geological Survey of Ireland, 13–16 (1861).
- <sup>14</sup> Woodward, A. S., *Catalogue of the fossil fishes in the British Museum (Natural History)*, 2, 292, plate VIII, figs 1–4 (1891).
- <sup>15</sup> Charlesworth, J. K., *Historical Geology of Ireland*, 180–4 (Oliver and Boyd, Edinburgh, 1963).
- <sup>16</sup> Miles, R. S., and Westoll, T. S., *Trans. R. Soc. Edinb.*, 67, 373–476 (1968).
- <sup>17</sup> Hills, E. S., *Q. Jl Geol. Soc. Lond.*, 88, 850–858 (1932).
- <sup>18</sup> Ritchie, A., *Palaontographica*, A143, 58–72 (1973).
- <sup>19</sup> Denison, R. H., *Feldiana, Geol.*, 11, 459–551 (1958).
- <sup>20</sup> Obruchev, D. V., *Fundamentals of Palaeontology. Class Placodermi XI* (English translation), 197–200 (Israeli Program for Scientific Translations, Jerusalem, 1967).
- <sup>21</sup> Miles, R. S., *Trans. R. Soc. Edinb.*, 68, 123–170 (1969).
- <sup>22</sup> Miles, R. S., *Phil. Trans. R. Soc.*, 263, 101–234 (1971).

# Unique and repetitive sequences in multiple genes for feather keratin

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*Embryonic chick feather keratins are a family of homologous polypeptide chains. The mRNA coding for these has been obtained in a pure state and transcribed into complementary DNA (cDNA) using the reverse transcriptase from avian myeloblastosis virus. Studies on the kinetics of hybridisation and reannealing of cDNA indicate that there are 25–35 different keratin mRNA species in the embryonic chick feather, and a total of 100–240 keratin genes in the chick genome. Each keratin gene contains both a unique and a repetitive sequence. It is proposed that the repetitive sequences are the keratin coding sequences and that the unique sequences correspond to untranslated regions.*

SINCE the original demonstration of highly repetitive, moderately repetitive and unique sequence classes in the nuclear DNA of higher organisms<sup>1–3</sup>, it has been established that most highly repetitive sequences fall into a distinct class of simple-sequence non-coding DNA (satellites) occurring in long uninterrupted blocks in the genome<sup>4,5</sup>. In contrast, the moderately repetitive DNA seems to be much more varied in function and organisation. Much of the moderately repetitive DNA seems to occur in short segments intimately interspersed with unique sequences<sup>1–3,6,7</sup>, and various models have attributed control functions to this class of DNA (refs 8–11). Whereas the structural genes for some (and presumably most) proteins occur in the unique DNA fraction<sup>12–14</sup>, moderately repetitive structural genes have also been identified. The genes for ribosomal RNA (r RNA) (refs 15 and 16), 5S RNA (ref. 17), tRNA (ref. 18) and histones<sup>19</sup> are moderately repetitive and occur as tandem repeats in the genome. Unique sequences are not, however, interspersed between these repetitive sequences and an example of a set of repetitive DNA sequences of known function, each directly linked to a unique sequence, has not previously been described.

As chick feather keratins consist of a large family of homologous proteins<sup>20–22</sup>, keratin genes must represent a family of non-identical moderately repetitive DNA sequences. The availability of highly purified feather keratin mRNA (refs 23–25) affords an opportunity to study the organisation of this family of genes. Feather keratin chains are the major protein products of the 14 day embryonic chick feather<sup>26</sup>. About 19–25 distinct keratin chains, differing in primary structure by multiple amino acid substitutions, have been identified in the

embryonic feather<sup>20,21</sup>. Additional keratin chains are present in adult feather tissues<sup>20</sup> indicating at least 30 homologous keratin genes in the chick genome<sup>22</sup>. These have presumably arisen by gene duplication and subsequent divergence.

A 12S RNA species isolated from 14 day embryonic chick feather polysomes<sup>23</sup> was released from polysomes as an mRNP complex by EDTA treatment<sup>24</sup>, bound selectively to cellulose<sup>23,24</sup> and coded only for keratin chains in the wheat embryo cell-free system<sup>25</sup>. Keratin mRNA migrated as one band of molecular weight 250,000 on formamide-acrylamide gels<sup>24</sup>, indicating an untranslated sequence, including poly(A), of about 500 bases in addition to the keratin-coding sequence of 300 bases. Since the keratin mRNA coded for at least four and probably all of the different keratin chains<sup>25</sup> it consists of a mixture of non-identical homologous keratin mRNAs of closely similar size.

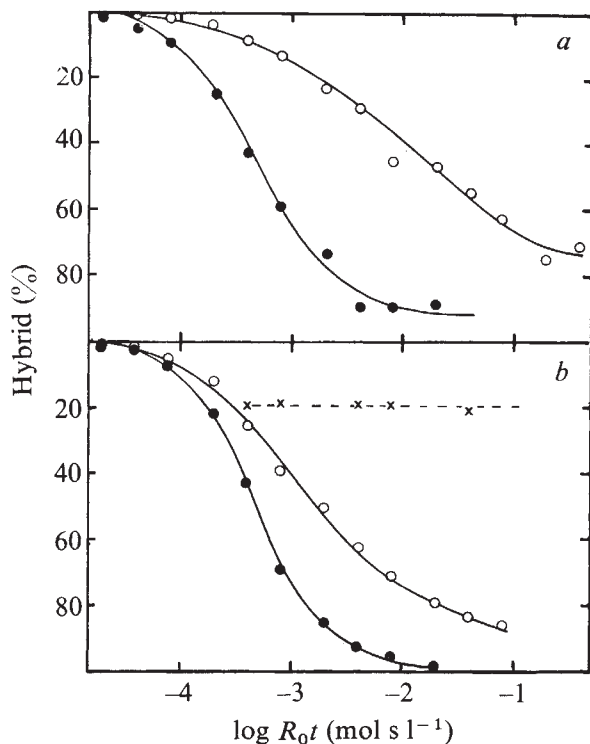
Keratin mRNA acted as an efficient template (ref. 22 and D. J. Kemp, and G. E. Rogers, unpublished) for the oligo(dT)-dependent synthesis of complementary DNA (cDNA) by the DNA polymerase of avian myeloblastosis virus (AMV), as reported for other mRNAs (refs 27–29). Keratin cDNA migrated as a major peak (about 70% total) of apparent molecular weight 170,000 on formamide-acrylamide gels (D. J. Kemp and G. E. Rogers, unpublished), demonstrating that most keratin cDNA molecules must contain sequences complementary to at least parts of both the keratin-coding sequences and untranslated sequences. I report hybridisation studies with keratin cDNA which demonstrate the presence of multiple keratin genes in the chick genome. Each keratin gene contains interspersed unique and repetitive sequences.

## Hybridisation of keratin mRNA to keratin cDNA

The kinetics of hybridisation of cDNA to excess unlabelled mRNA are dependent on the sequence complexity of mRNA. Comparison of the kinetics of hybridisation with those of a kinetic standard (for example, rabbit globin mRNA-cDNA; refs 30–32) allows an estimate of the complexity of keratin mRNA.

As determined by resistance of the hybrids to the single-strand specific nuclease S1 (refs 33–35), rabbit globin mRNA hybridised to globin cDNA in a sharp transition (Fig. 1a) with a mid point ( $R_{0.5}$ ) of  $4 \times 10^{-4}$  mol s l<sup>-1</sup> (refs 30–32). Keratin mRNA hybridised to keratin cDNA in a broad transition (Fig. 1a) with a  $R_{0.5}$  of about  $1 \times 10^{-2}$  mol s l<sup>-1</sup>. The kinetics cannot be ascribed to non-mRNA impurities in the preparations as they were reproducible using hatches of keratin mRNA which was transcribed at efficiencies as high as 43% of the theoretical maximum<sup>27–29</sup>. In addition, the keratin mRNA





**Fig. 1** Hybridisation of keratin and globin mRNA and poly(rA) to cDNA. mRNAs were prepared from EDTA-treated polyosomes as described<sup>24</sup>. cDNA was synthesised in a system (50  $\mu$ l) containing 50 mM Tris-HCl, pH 8.3, 8 mM dithiothreitol, 8 mM MgCl<sub>2</sub>, 0.66 mM dATP, dGTP, dTTP, 0.1 mM <sup>3</sup>H-dCTP (specific activity 26.2 mCi  $\mu$ mol<sup>-1</sup>), 100  $\mu$ g ml<sup>-1</sup> actinomycin D, 0.2–1.0  $\mu$ g mRNA, 0.1  $\mu$ g dT<sub>12–18</sub> and AMV-DNA polymerase, incubated for 1 h at 37°C, treated with 0.3 M NaOH and purified on Sephadex G-50 in 0.1 M NH<sub>4</sub>HCO<sub>3</sub> (ref. 28). The high molecular weight fraction (average about 170,000) of keratin cDNA, obtained by alkaline sucrose gradient sedimentation, was used for all experiments. Hybridisations were at 60°C for 4 h in 0.18 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl, 0.05% SDS, pH 7.0 (NETS). The reaction mixtures (100  $\mu$ l) contained 5,000 c.p.m. keratin or globin cDNA (transcribed at 27 and 60% efficiency, respectively in the experiment shown) and varying amounts of mRNA. For nuclease SI assays, 10  $\mu$ l aliquots were diluted into 150  $\mu$ l of 0.03 M Na-acetate, 0.05 M NaCl, 0.001 M ZnSO<sub>4</sub>, 5% glycerol, pH 4.6 (assay buffer) containing 12  $\mu$ g of sonicated, heat-denatured calf thymus DNA, incubated with or without nuclease SI (four units; purified through step four<sup>25</sup>; at 45°C for 30 min, TCA-precipitated and counted. HAP assays (on aliquots of the same reaction mixture shown in (a)) were as described<sup>3</sup> at 60°C in 0.18 M Na-phosphate (Na<sup>+</sup> molarity=0.18; pH 7.0). Where appropriate the results were normalised<sup>6</sup> to remove background and facilitate comparisons; the normalisation factor is shown in parenthesis for each curve; 0% indicates that a curve was not normalised. *a*, Nuclease SI assays: ○, keratin mRNA-cDNA (0%); ●, globin mRNA-cDNA (5%). *b*, HAP assays: ○, keratin mRNA-cDNA (10%); ●, globin mRNA-cDNA (20%); ×, poly(rA)-keratin cDNA (10%).

used here contained only one band on formamide gels and coded only for keratins in the wheat embryo system<sup>24,25</sup>.

When hybridisation of keratin mRNA to cDNA was assayed on hydroxyapatite (HAP), a major transition was observed (Fig. 1b) with a  $R_0t_{1/2}$  of  $9 \times 10^{-4}$  mol s l<sup>-1</sup>, about ten times faster than the same reaction assayed with nuclease SI. In contrast, the kinetics of hybridisation of globin mRNA-cDNA were identical whether assayed on HAP (Fig. 1b) or with nuclease SI (Fig. 1a). The major transition of keratin mRNA cannot be explained by interaction of cDNA with the poly(A) segment of keratin mRNA as only about 20% of keratin cDNA molecules formed hybrids with poly(rA) (Fig. 1b), and was not caused by self-complementarity of the cDNA since incubation of cDNA in the absence of mRNA for 0–4 h resulted in 10% binding to HAP.

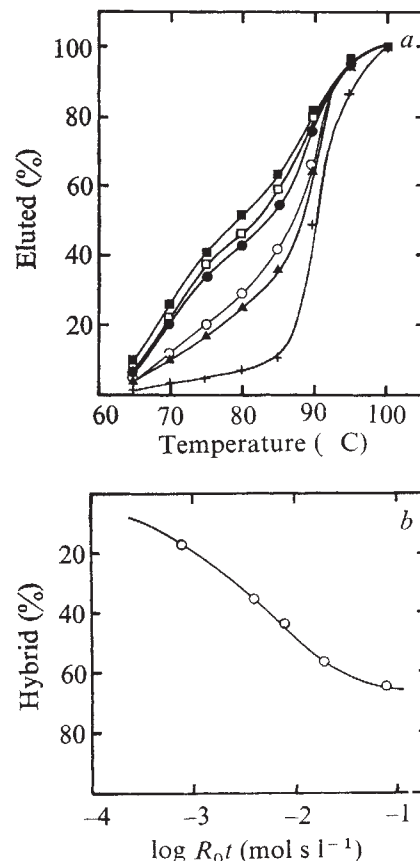
The thermal stabilities of keratin mRNA-cDNA hybrids changed as a function of  $R_0t$  (Fig. 2a). At least two com-

ponents with  $T_m$  of about 72° and 90° were evident. Hybrids which eluted from HAP at greater than 80° formed with a  $R_0t_{1/2}$  of about  $6\text{--}7 \times 10^{-3}$  mol s l<sup>-1</sup> (Fig. 2b), a value similar to the rate observed using nuclease SI (Fig. 1a). In contrast, only one major component of high thermal stability was present in globin mRNA-cDNA hybrids (Fig. 2a), demonstrating that the low thermal stability of keratin hybrids was not caused by their degradation under the incubation conditions used here.

### Reannealing of keratin cDNA with chick erythrocyte nuclear DNA

The reiteration frequency of sequences in cDNA preparations can be estimated by annealing cDNA with a vast excess of chromosomal DNA<sup>13,14,36–38</sup>. As determined by nuclease SI assays in low salt buffer (Fig. 3a) chick DNA (labelled either with <sup>125</sup>I *in vitro*<sup>39</sup> or with <sup>32</sup>P *in vivo*) reannealed with a mid-point ( $C_0t_{1/2}$ ) of about  $1 \times 10^3$  mol s l<sup>-1</sup> (ref. 38). Keratin cDNA reannealed to a vast excess of chick DNA with a  $C_0t_{1/2}$  of about  $4 \times 10^2$  mol s l<sup>-1</sup> (Fig. 3a). As determined by nuclease SI assays in high salt buffer (Fig. 3b) <sup>125</sup>I-labelled chick DNA reannealed at a similar rate to that determined in the low salt assays but seemingly to a greater extent. Assayed under these conditions keratin cDNA gave a broad curve (Fig. 3b) suggesting two transitions with  $C_0t_{1/2}$  values of about  $4 \times 10^1$  and  $1 \times 10^3$  mol s l<sup>-1</sup>, respectively.

**Fig. 2** Thermal stabilities of mRNA-cDNA hybrids. Aliquots as described in Fig. 1 were diluted into 0.18 M Na-phosphate (pH 7.0) containing 100  $\mu$ g partially re-associated calf thymus DNA and loaded on to HAP at 60°C. Hybrids which could not be eluted at 60°C were melted by raising the temperature in 5°C increments. Two 2.5 ml washes (0.18 M Na-phosphate) were collected at each temperature and TCA-precipitated. *a*, Keratin mRNA-cDNA hybrids, after incubation to  $R_0t$  values (mol s l<sup>-1</sup>) of: ■,  $8 \times 10^{-4}$ ; □,  $4 \times 10^{-3}$ ; ●,  $8 \times 10^{-3}$ ; ○,  $2 \times 10^{-2}$ ; ▲,  $8 \times 10^{-2}$ ; +, globin mRNA-cDNA hybrids,  $8 \times 10^{-4}$ . *b*, Rate of formation of keratin mRNA-cDNA hybrids eluting from HAP at greater than 80°C. The normalised percentage cDNA bound to HAP at 60°C (data from Fig. 1b) was multiplied by the percentage of this bound cDNA which eluted at greater than 80°C (data from Fig. 2a), for each  $R_0t$  value shown.

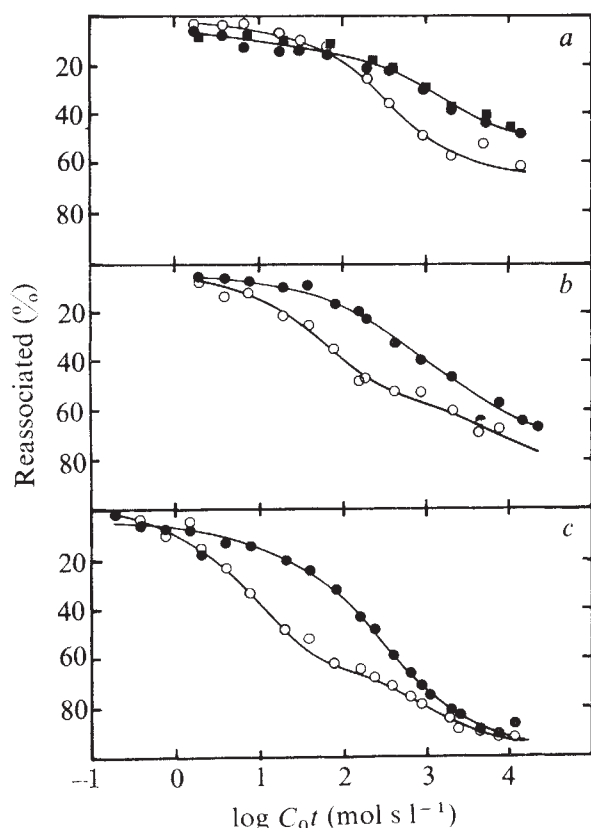


When assayed on HAP (Fig. 3c) chick DNA reannealed with a major transition at a  $C_0t_1$  of about  $4 \times 10^2 \text{ mol s l}^{-1}$ , a value compatible with estimates of the size of the chick genome<sup>3,14,40,41</sup>. Keratin cDNA gave two distinct transitions (Fig. 3c) with  $C_0t_1$  values of  $7 \text{ mol s l}^{-1}$  (about 60% total) and  $5 \times 10^2 \text{ mol s l}^{-1}$  (about 30% total).

The thermal stabilities of the keratin cDNA-DNA duplexes (Fig. 4a) changed with  $C_0t$  in a manner similar to but more marked than the mRNA-cDNA hybrids (Fig. 2a). Two components with  $T_m$  of about  $73^\circ$  and  $93^\circ$  were evident. Duplexes which eluted from HAP at greater than  $85^\circ$  (Fig. 4b) formed with a  $C_0t_1$  of about  $6 \times 10^2 \text{ mol s l}^{-1}$ . From the data in Figs 3b, 4a and b it can be calculated that of the keratin cDNA duplexes with  $T_m$  lower than  $85^\circ$  at a  $C_0t$  of  $6 \times 10^1 \text{ mol s l}^{-1}$ , more than 70% of these same molecules have  $T_m$  greater than  $85^\circ$  at a  $C_0t$  of  $1.2 \times 10^4 \text{ mol s l}^{-1}$ .

### Interspersed unique and repetitive sequences in keratin genes

The striking features of the results are the greater apparent rates of formation of keratin cDNA duplexes when assayed with HAP compared with nuclease S1 and the progressive increase in average thermal stability of the duplexes with



**Fig. 3** Reassociation of keratin cDNA with excess chick erythrocyte nuclear DNA. Chick erythrocyte DNA, prepared as described<sup>31</sup>, was sonicated (mean length  $0.16 \mu\text{m}$ ). Reaction mixtures (in NETS) contained  $9.1 \text{ mg ml}^{-1}$  sonicated DNA plus about  $35,000 \text{ c.p.m. ml}^{-1}$  keratin cDNA,  $^{125}\text{I}$ -chick erythrocyte-DNA (ref. 39) (specific activity  $1 \times 10^5 \text{ c.p.m. } \mu\text{g}^{-1}$ ), or  $^{32}\text{P}$ -labelled chick embryo DNA, and were denatured ( $100^\circ \text{C}$  for 5 min) before incubation at  $60^\circ \text{C}$  to the  $C_0t$  values shown. For nuclease S1 assays in low salt buffer,  $50 \mu\text{l}$  aliquots were diluted into assay buffer and incubated with or without nuclease S1 (150 units) for 30 min at  $45^\circ \text{C}$ . Nuclease S1 assays in high-salt were identical except that the assay buffer contained  $0.3 \text{ M}$  NaCl and incubation was at  $37^\circ \text{C}$ . HAP assays were as in Fig. 1b. After S1 digestion or HAP fractionation, all samples for any one curve were made up to the same final DNA concentration before TCA precipitation to avoid errors resulting from differential quenching. Normalisation factors are shown as for Fig. 1. *a*, Nuclease S1 assays in low-salt:  $\bullet$ ,  $^{125}\text{I}$ -DNA (0%);  $\blacksquare$ ,  $^{32}\text{P}$ -DNA (0%);  $\circ$ , keratin cDNA (0%). *b*, Nuclease S1 assays in high-salt:  $\bullet$ ,  $^{125}\text{I}$ -DNA (14%);  $\circ$ , keratin cDNA (0%). *c*, HAP assays:  $\bullet$ , DNA ( $A_{260}$ ) (12%);  $\circ$ , keratin cDNA (10%).

increasing  $C_0t$ . The data can be explained if there are multiple homologous keratin genes in the chick genome each containing two distinct sequences of approximately equal length, namely (1) a similar but non-identical sequence common to all the keratin genes ('reiterated sequence'), covalently attached to (2) a sequence so different in base sequence to that of any other keratin gene ('unique sequence') that it seems to be unique in the genome under the experimental criteria used here.

The rapid transitions of keratin cDNA observed on HAP (compared with nuclease S1 in low salt conditions) can be explained as follows. The reiterated sequences cross-hybridise early in the reaction (Figs 1b and 3c), forming duplexes which bind the entire molecule to HAP (ref. 3). The unpaired unique regions would, however, be completely susceptible to nuclease S1. The low thermal stability of these duplexes indicates that about 20% of the base pairs are mismatched<sup>43</sup>. The slow formation of duplexes of high thermal stability (Figs 2b and 4b) would result from hybridisation of the unique regions to their exact complements, forming concatenates of the early structures. These duplexes would be stable to nuclease S1.

On this model, two distinct transitions would be expected after assaying hybridisation with nuclease S1, the faster corresponding to the rapidly formed duplexes of the reiterated sequences and the slower corresponding to the unique sequences. This was not observed when nuclease S1 assays were carried out in low salt (Figs 1a and 3a). The single transition observed, however, occurred over three decades of  $R_0t$  (Figs 1a and 3a), a sure indication of heterogeneity<sup>3</sup>. Under high salt conditions in the S1 assays two distinct transitions were observed with keratin cDNA-DNA duplexes (Fig. 3b). The extent of this early transition (Fig. 3b) suggests that the reiterated sequences occupy about 50% (that is, about 250–300 bases) of the cDNA molecules.

The difference in S1-resistance of early keratin cDNA-DNA duplexes in low and high salt (compare Fig. 3a and b) can be explained if the mismatched duplexes are degraded to an extent of about 50% in low salt and are completely resistant in high salt. Therefore, although two distinct transitions are not apparent in Figs 1a and 3a, the first 20–30% of the observed transition can be attributed to mismatched duplexes of the reiterated region which are partially degraded by nuclease S1, resulting in a final extent of only 70–80% resistance of the hybrids to nuclease S1 (Fig. 1a). The presence of high-salt concentrations did not protect cDNA-mRNA hybrids against degradation by nuclease S1 (data not shown). The smaller slow transitions of keratin cDNA observed on HAP (Figs 1b and 3c) could result from thermal scission, incomplete transcription, impurities in mRNA, or a combination of these factors.

### Estimation of the number of molecular species in embryonic chick keratin mRNA

The kinetic complexity of the unique sequences in keratin mRNA estimated by the nuclease S1 procedure (Fig. 1a) was about 25 times that of globin mRNA, and estimated by the rate of formation of hybrids eluting from HAP at greater than  $80^\circ$  (Fig. 2b) was about 18 times that of globin mRNA. Since the  $\alpha$  and  $\beta$  chains of rabbit globin mRNA and cDNA do not cross hybridise<sup>30–32</sup> the complexity of globin mRNA is about 1,100 bases, giving an apparent complexity of about 20,000–28,000 bases for keratin mRNA. Since these unique sequences only occupy about half of the mRNA, this adjusts to 10,000–14,000 bases when corrected for the actual concentration of unique sequences instead of total mRNA, equivalent to 25–35 different unique sequences about 400 bases long. This estimate of 25–35 different mRNA species is compatible with the minimal number of 19 to 25 keratin chains<sup>21,22</sup> detected in embryonic chick feather keratin.

### Estimation of the number of keratin genes in the chick genome

Keratin cDNA reannealed to chick DNA about two to three



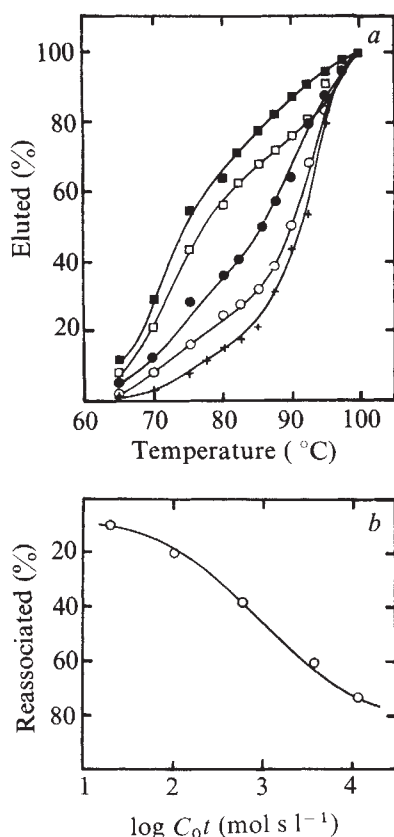


Fig. 4 Thermal stabilities of keratin cDNA-DNA duplexes. Reaction mixtures as in Fig. 3 were treated as in Fig. 2, except that carrier DNA was not added until after fractionation on 2.0 ml HAP columns. *a*, Keratin cDNA-DNA duplexes after incubation to  $C_0t$  values (mol s l<sup>-1</sup>) of: ■,  $1.9 \times 10^1$ ; □,  $1.0 \times 10^2$ ; ●,  $5.7 \times 10^2$ ; ○,  $3.06 \times 10^3$ ; +,  $1.15 \times 10^4$ . *b*, Rate of formation of keratin cDNA-DNA duplexes eluting from HAP at greater than 85 °C, calculated from the data of Figs 3c and 4a as described for Fig. 2b.

times faster than the unique sequence fraction of chick DNA as determined by the nuclease S1 procedure in low salt, indicating that the unique regions are reiterated two or three times. The strong dependence of the kinetics on the salt concentration in the nuclease S1 assays suggests that this could be an overestimate. This interpretation is supported by the kinetics of formation of keratin cDNA-DNA duplexes eluting from HAP at greater than 85 °C (Fig. 4b), which indicate that the unique sequences occur only once in the haploid genome.

The reiterated sequences in keratin mRNA hybridised to keratin cDNA with a  $R_0t_0$  of  $9 \times 10^{-4}$  mol s l<sup>-1</sup> when measured on HAP (Fig. 1b), about four times slower than the rate expected for a single perfectly complementary species. Mismatching of about 20% (ref. 42) would lower the reaction rate by a factor of about four<sup>42</sup>, giving a corrected apparent complexity of about one for the reiterated sequence. This implies that most if not all keratin mRNA species share the sequence.

The reiterated sequences reannealed to chick DNA about 60 times as fast as the unique sequence fraction of chick DNA when determined on HAP (Fig. 3c) and about 25 times as fast when assayed with nuclease S1 in high salt (Fig. 3b). When corrected by the factor of four for the reduction in rate caused by mismatching, the reiteration frequency of this sequence seems to be 100–240. If, however, the low  $T_m$  of duplexes of these regions is a consequence of the sequences being very short rather than poorly matched, the factor of four may not apply and the estimate may be too high. This would not invalidate the conclusion that there are two distinct sequence classes present in each keratin gene.

## Nature of the unique and reiterated sequences in keratin genes

The limited available sequence data<sup>21</sup> indicate that individual embryonic chick feather keratin chains differ from each other in amino acid sequence by a maximum of about 8% and that most of these differences are the result of single base changes. In the conditions used here, stable hybrids form between mouse globin cDNA and rabbit globin mRNA (ref. 43), although mouse and rabbit globin chains differ by 19% in amino acid sequence. Keratin structural genes should therefore cross hybridise with each other unless mutations which do not change the amino acid specified (relative to those that do change the amino acid specified) occur in keratin structural genes much more frequently than in globin structural genes. It is therefore likely that the reiterated sequences are the keratin structural genes, that there are about 100–240 of these in the haploid chick genome, that on average they have diverged from each other by about 20% in base sequence, and that many of them are not expressed, or expressed at a very low level in the embryonic feather.

If this interpretation is correct, it follows that the unique sequences are the untranslated regions and that these have diverged at a much greater rate, to the extent that each seems to be unique in the haploid genome. A similar situation has been observed in the case of sea-urchin histone genes<sup>44</sup>. In this case the spacer sequences are also thought to have diverged at a greater rate than the histone structural genes<sup>44</sup>. This also seems to be true for the spacer sequences of ribosomal genes<sup>45</sup>. Preliminary results indicate that shorter keratin cDNA molecules obtained using *Escherichia coli* DNA polymerase I to copy keratin mRNA contain only the unique sequences, suggesting that the unique sequences are localised toward the 3' end of keratin mRNA.

Recent studies indicate that most eukaryotic mRNAs are transcribed from unique sequences<sup>12–14,30,37,38,46,47</sup>, although a small fraction which seems to be transcribed from repetitive sequences has also been reported<sup>30,46–48</sup>. In contrast to the situation in keratin mRNA, however, these repetitive and single-copy transcripts are not covalently interspersed<sup>46,47</sup>. Whereas the genes for duck globin seem to be adjacent to repetitive sequences in the genome<sup>49</sup>, keratin genes seem to be the first example of repetitive sequences of known function which are interspersed with unique sequences. The occurrence of both unique and repetitive sequences in keratin genes should greatly facilitate studies on their arrangement in the chick genome.

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- 1 Waring, M. J., and Britten, R. J., *Science*, **154**, 791–794 (1966).
- 2 Bolton, E. T., et al., *Yb. Carnegie Instn. Wash.*, **64**, 316–332 (1966).
- 3 Britten, R. J., and Kohne, D. E., *Science*, **161**, 529–540 (1968).
- 4 Southern, E. M., *Nature*, **227**, 794–798 (1970).
- 5 Peacock, W. J., et al., *Cold Spring Harb. Symp. quant. Biol.*, **38**, 405–416 (1973).
- 6 Davidson, E. H., Hough, B. R., Amenson, C. S., and Britten, R. J., *J. molec. Biol.*, **77**, 1–23 (1973).
- 7 Graham, D. E., Neufeld, B. R., Davidson, E. H., and Britten, R. J., *Cell*, **1**, 127–136 (1974).
- 8 Britten, R. J., and Davidson, E. H., *Science*, **165**, 349–357 (1969).
- 9 Crick, F. H., *Nature*, **234**, 25–27 (1971).
- 10 Georgiev, G. P., *J. theor. Biol.*, **25**, 473–490 (1969).
- 11 Elder, D., *J. theor. Biol.*, **39**, 673–675 (1973).
- 12 Suzucki, Y., Gage, L. P., and Brown, D. D., *J. molec. Biol.*, **70**, 637–649 (1972).
- 13 Bishop, J. O., and Rosbash, M., *Nature new Biol.*, **241**, 204–207 (1973).
- 14 Harris, S. E., Means, A. R., Mitchell, W. M., and O'Malley, B. W., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3776–3780 (1973).
- 15 Birnstein, M. L., Wallace, H., Sirlin, J. L., and Fischberg, M., *Natn. Cancer Inst. Monogr.*, **23**, 431–447 (1966).
- 16 Brown, D. D., and David, I. B., *Science*, **160**, 272–280 (1968).
- 17 Brown, D. D., Wensink, P. C., and Jordan, E., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 3175–3179 (1971).
- 18 Clarkson, S. G., Birnstein, M. L., and Serra, V., *J. molec. Biol.*, **79**, 391–410 (1973).
- 19 Kedes, L. H., and Birnstein, M. L., *Nature new Biol.*, **230**, 165–169 (1971).
- 20 Kemp, D. J., and Rogers, G. E., *Biochemistry*, **11**, 969–975 (1972).
- 21 Walker, I. D., thesis, Univ. Adelaide (1974).

- <sup>22</sup> Kemp, D. J., Walker, I. D., Partington, G. A., and Rogers, G. E., in *The Eukaryote Chromosome* (edit. by Peacock, W. J., and Brock, R. D.), (ANU, Canberra, in the press).
- <sup>23</sup> Partington, G. A., Kemp, D. J., and Rogers, G. E., *Nature new Biol.*, **246**, 33–36 (1973).
- <sup>24</sup> Kemp, D. J., Partington, G. A., and Rogers, G. E., *Biochem. biophys. Res. Commun.*, **60**, 1006–1014 (1974).
- <sup>25</sup> Kemp, D. J., Schwinghamer, M. W., and Rogers, G. E., *Molec. Biol. Rep.*, **1**, 441–446 (1974).
- <sup>26</sup> Kemp, D. J., Dyer, P. Y., and Rogers, G. E., *J. cell. Biol.*, **62**, 114–131 (1974).
- <sup>27</sup> Ross, J., Aviv, H., Scolnick, E., and Leder, P., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 264–268 (1972).
- <sup>28</sup> Verma, I. M., Temple, G. F., Fan, H., and Baltimore, D., *Nature new Biol.*, **235**, 163–167 (1972).
- <sup>29</sup> Kacian, D. L., et al., *Nature new Biol.*, **235**, 167–169 (1972).
- <sup>30</sup> Bishop, J. O., Morton, J. G., Rosbash, M., and Richardson, M., *Nature*, **250**, 199–203 (1974).
- <sup>31</sup> Kacian, D. L., et al., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 186–18890 (1973).
- <sup>32</sup> Housman, D., Forget, B. G., Skoultschi, A., and Benz, E. J., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1809–1813 (1973).
- <sup>33</sup> Ando, T., *Biochim. biophys. Acta.*, **114**, 158–168 (1966).
- <sup>34</sup> Sutton, W. D., *Biochim. biophys. Acta.*, **240**, 522–531 (1971).
- <sup>35</sup> Vogt, V. M., *Eur. J. Biochem.*, **33**, 192–200 (1973).
- <sup>36</sup> Melli, M., Whitfield, C., Rao, K. V., Richardson, M., and Bishop, J. O., *Nature new Biol.*, **231**, 8–12 (1971).
- <sup>37</sup> Harrison, P. R., Hell, A., Birnie, G. D., and Paul, J., *Nature*, **239**, 219–221 (1972).
- <sup>38</sup> Sullivan, D., et al., *J. biol. Chem.*, **248**, 7530–7539 (1973).
- <sup>39</sup> Tereba, A., and McCarthy, B. J., *Biochemistry*, **12**, 4675–4679 (1973).
- <sup>40</sup> Laird, C. D., *Chromosoma*, **32**, 378–406 (1971).
- <sup>41</sup> Mirsky, A. E., and Ris, H., *J. gen. Physiol.*, **34**, 451 (1951).
- <sup>42</sup> Bonner, T. I., Brenner, D. J., Neufeld, B. R., and Britten, R. J., *J. molec. Biol.*, **81**, 123–135 (1973).
- <sup>43</sup> Gummerson, K. S., and Williamson, R., *Nature*, **247**, 265–267 (1974).
- <sup>44</sup> Birnstiel, M., Telford, J., Weinberg, E., and Stafford, D., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2900–2904 (1974).
- <sup>45</sup> Brown, D. D., Wensink, P. C., and Jordan, E., *J. molec. Biol.*, **63**, 57–73 (1972).
- <sup>46</sup> Goldberg, R. B., Galau, G. A., Britten, R. J., and Davidson, E. H., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3516–3520 (1973).
- <sup>47</sup> Klein, W. H., Murphy, W., Attardi, G., Britten, R. J., and Davidson, E. H., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1785–1789 (1974).
- <sup>48</sup> Rabbitts, T. H., Bishop, J. O., Milstein, C., and Brownlee, G. G., *FEBS Lett.*, **40**, 157–160 (1974).
- <sup>49</sup> Dina, D., Crippa, M., and Beccari, E., *Nature new Biol.*, **242**, 101–105 (1973).
- <sup>50</sup> Bishop, J. O., and Freeman, K., *Cold Spring Harb. Symp. quant. Biol.*, **38**, 707–716 (1973).
- <sup>51</sup> Marushige, K., Brutlag, D., and Bonner, J., *Biochemistry*, **7**, 3149–3155 (1968).

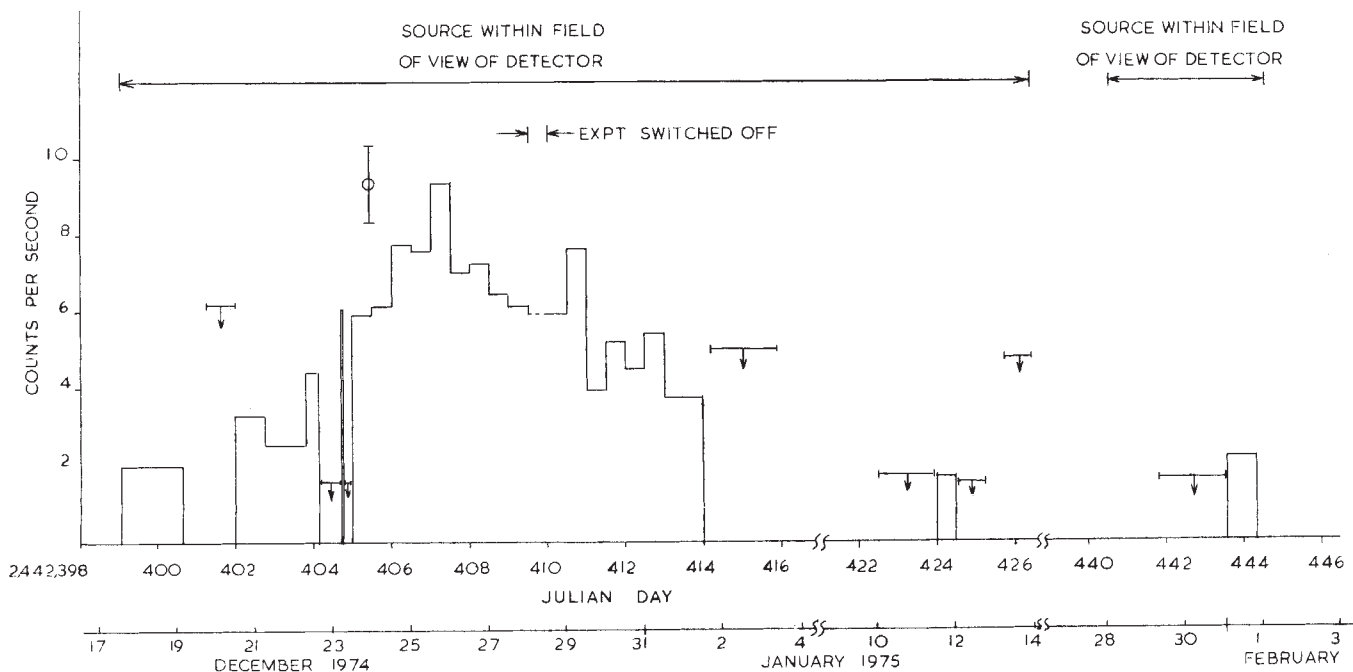
# letters to nature

## Variable X-ray source near Cen X-3

THE satellite Ariel V, launched on October 15, 1974, as part of the US-UK collaborative space programme, contains a rotation modulation collimator experiment designed to measure the positions of X-ray sources, which was built by the Mullard Space Science Laboratory. The experiment responds to X rays in the quantum energy range 3.1–9.3 keV, the area of the proportional counters being 256 cm<sup>2</sup> which, allowing for grid transmission, counter efficiency and so on, leads to an effective area of 102 cm<sup>2</sup> at 6.0 keV. The pitch-to-separation ratio of

the collimator grids is 112, corresponding to a full width at half maximum (FWHM) of the image of a point source of 15' (ref. 1). The spin axis was manoeuvred to point towards a number of sources in succession in the Centaurus region, including the important binary source Cen X-3, from December 17, 1974 to January 31, 1975, to carry out position and spectrum determinations. Soon after arriving at the first pointing direction in the sequence, a correlation map produced by overlaying data from orbits obtained on December 17–19 showed the presence of a new source not listed in the Uhuru (3U) catalogue, which we have designated A1118-61 from the position given below. The field of view of the experiment is 17°

**Fig. 1** Counting rate of variable source in Centaurus region as a function of time after subtraction of background. The symbol circle with a vertical bar indicates typical  $\pm 1\sigma$  values near the maximum intensity; as noted in the text this arises partly from fluctuations in source strength on successive orbits. The standard deviation of the fluctuations in background level is one-half of this or less. The variation in the magnitude of the upper limits is because of changes in the distance of the source from the spin axis, except on December 20 for which the data were of relatively poor quality.





FWHM, so that the light curve could be followed over an interval of 46 d. The light curve is given in Fig. 1 in units of counting rate in our detector, which can be converted approximately to Uhuru counts by multiplication by 8. In the initial and final periods of observation, the source was rather weak, and could be detected at a satisfactory significance only by overlaying orbits, with a consequent reduction in time resolution. The latter improved to one orbit (101 min) when the source was near its maximum brightness. We have, for clarity, averaged the intensity over 12 h in this period, but significant intensity variations, exceeding those attributable to counting statistics, were observed in the one-orbit averages. Where an upper limit only is given, it corresponds to three standard deviations of the background level.

Both at the beginning and at the end of our observation period the emission from the source was detectable. It is possible that the event we observed is a rather strong flare of the kind observed in Sco X-1. On the other hand, if it is assumed that the emission rose to the level we observed immediately before our observations, and that it decayed further afterwards, then the X-ray light curve strongly resembles that shown at optical wavelengths by some moderately fast novae, which may exhibit a 'pre-maximum halt', a dip and a rise to the maximum. The structure in the light curve on JD 2, 442, 404–5 is real, the emission before and after the sharp peak being at a low level, on which we could place only upper limits. The intensity change on either side of the dip exceeds  $5\sigma$ , and would correspond to the dip in the nova light curve. The X-ray light curve from this source differs considerably from that of Cen X-4 (ref. 2) which showed no pre-maximum dip. At times when the pointing direction was sufficiently close, another experiment on Ariel V obtained data on the short-period fluctuations which are described in the accompanying letter<sup>3</sup>.

We have obtained a position for this source. Our experiment is most accurate when another X-ray source of accurately known position is simultaneously in the field of view and we hoped to use Cen X-3 for this purpose. Unfortunately from December 17 to January 10, Cen X-3 was in an 'extended low'. After this its intensity increased only rather slowly and by this time the brightness of the new source had diminished considerably. We were, however, able to obtain four simultaneous observations of the two sources. We also observed two other Uhuru sources, 3U1223–62 and 3U1254–69, and a second, new, variable source. By considering all the simultaneous observations of these sources with one another and with Cen X-3, we have been able to improve this estimate of the position, with the final result RA 11 h 18 min 59  $\pm$  13 s, declination  $-61^\circ 35.3' \pm 1.8'$ . These are 1950 coordinates, the errors being  $\pm 2\sigma$ . The position used for Cen X-3 was RA 11h 19.05 min, declination  $-60^\circ 21'$  (ref. 4). We note that our observations place both the 3U sources outside of and to the north-east of their 3U error boxes, though by varying amounts, which may indicate a systematic error in our results which is not allowed for in the position error we have quoted. We have looked for such a systematic error and failed to find any direct evidence for it. On the basis of the position of 3U1254–69, which has the smallest error box, we estimate the maximum value of any error at  $4'$ , such that the source would lie to the south-west of the position given.

Although the X-ray emission may now have decayed below the value we first observed, we suggest that optical observation of possible candidate stars may still be worthwhile in view of the periodicity described in ref. 3.

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- <sup>1</sup> Willmore, A. P., *Mon. Not. R. astr. Soc.*, **147**, 387 (1970).
- <sup>2</sup> Evans, W. D., Belian, R. D., and Connor, J. P., *Acrophys. J. Lett.*, **159**, L57 (1970).
- <sup>3</sup> Ives, J. C., Sanford, P. W., and Belz Burnell, S. J., *Nature*, **254**, 578 (1975).
- <sup>4</sup> Parkinson, J. H., Mason, K. O., and Sanford, P. W., *Nature*, **249**, 746–747 (1974).

## Observations of a transient X-ray source with regular periodicity of 6.75 min

DURING planned observations, by the satellite Ariel V, of the X-ray source Cen X-3 (3U1118-60), a previously unreported source nearby was detected by the collimated proportional counter of 100 cm<sup>2</sup> provided by the Mullard Space Science Laboratory. The source was monitored over a period of 39 d between December 19, 1974, and January 27, 1975. Here we report measurements of the light curve, spectrum and of a regular variability with period of  $6.755 \pm 0.010$  min. The position of the source is given in the accompanying letter<sup>1</sup>.

The intensity of the source is shown plotted against time in Fig. 1 for the period December 19, 1974 to January 1, 1975, during which time Cen X-3 was in an extended low state. The first observations on December 19–20, 1974, indicated a weak source of  $0.006$  photon cm<sup>-2</sup> s<sup>-1</sup> keV<sup>-1</sup> in the energy range 3–30 keV. The intensity increased by a factor of four during the period December 20–25, 1974, peaking at a strength of  $0.02$  photon cm<sup>-2</sup> s<sup>-1</sup> keV<sup>-1</sup>. Subsequently, the intensity decayed with an e-folding time of approximately 7 d. The intensity at the time of the final observation on January 27, 1975, had fallen to  $0.004$  photon cm<sup>-2</sup> s<sup>-1</sup> keV<sup>-1</sup>. This and other data after January 1, 1975, were taken during Cen X-3 binary off periods.

The last two points on Fig. 1a show an increase in intensity and are coincident with a predicted<sup>2</sup> binary transition of Cen X-3 from off to on. Immediately after this, the spacecraft moved away from Cen X-3 for 7 d. It seems likely that this marks the first turn-on of Cen X-3 after the extended low.

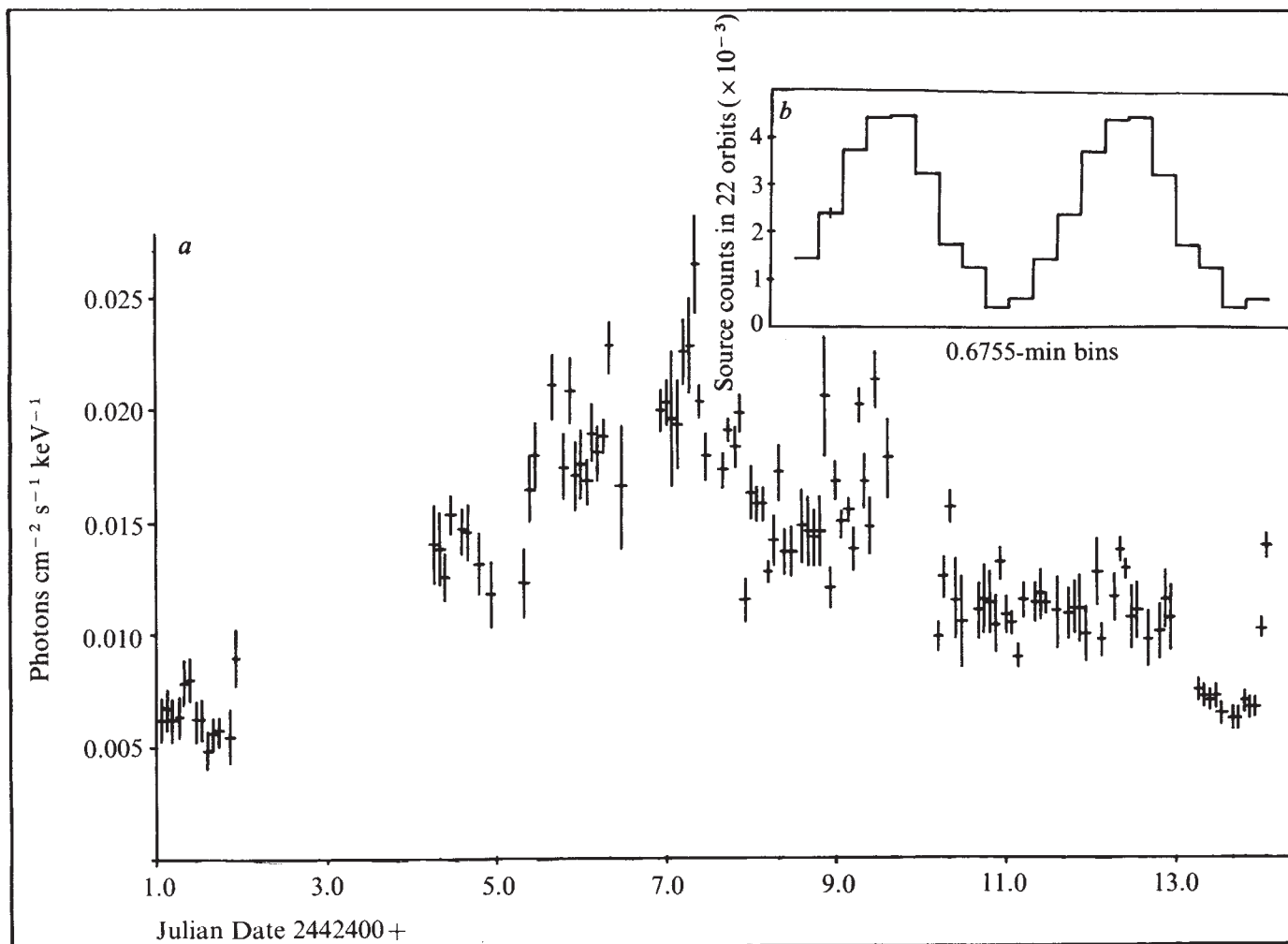
Spectra of the source have been obtained with 32 pulse-amplitude channels analysing the output from the proportional counter in two energy ranges, 1.5–15 keV and 3.0–30 keV. The data discussed here refer predominantly to the former.

Table 1 Best-fit parameters

| Phase           | Coefficient     | Power           | $N_H$                          | $\chi^2$ |
|-----------------|-----------------|-----------------|--------------------------------|----------|
| Before          | $0.11 \pm 0.02$ | $1.10 \pm 0.07$ | $(5.5 \pm 0.8) \times 10^{22}$ | 3.2      |
| During increase | $0.21 \pm 0.03$ | $1.07 \pm 0.07$ | $(6.6 \pm 0.8) \times 10^{22}$ | 3.2      |
| At maximum      | $0.22 \pm 0.03$ | $0.91 \pm 0.05$ | $(5.8 \pm 0.6) \times 10^{22}$ | 5.3      |
| During decay    | $0.22 \pm 0.03$ | $1.14 \pm 0.05$ | $(5.9 \pm 0.6) \times 10^{22}$ | 4.7      |

Spectra have been obtained from the different regions of the light curve—before and during the increase, at maximum and during the decay. In each case, the data are best fitted with a power law spectrum rather than an exponential or blackbody spectrum. No such simple model spectrum satisfactorily describes the data, however. The best-fit parameters are shown in Table 1, the errors being ( $\chi^2 + 1$ ) values.

Except for a marginally significant flattening of the spectrum at peak intensity, the spectral shape above 4 keV is constant



**Fig. 1a** Intensity of the source averaged over one orbit (1.6 hours), as a function of time (1 day  $\approx$  14 orbits). Errors shown are statistical. There may be an additional uncertainty of up to 6% caused by errors in the spacecraft attitude solutions. **b** 6.755-minute period light curve of the source. The error shown is statistical.

throughout the observations. The low energy spectrum, below 4 keV, shows a photon deficiency, independent of source model, indicating considerable absorption—amounting to  $6 \times 10^{22} N_H$  atoms  $\text{cm}^{-2}$ , assuming the abundances of Brown and Gould<sup>2</sup>. This value did not change during the observation. Measurements of 21-cm emission<sup>3</sup> give a column density to the edge of the Galaxy, in the direction of this source, of  $1.2 \times 10^{22}$  atoms. We conclude, therefore, that there is significant absorption from material local to the source.

The source intensity was measured on December 31, 1974, with a time resolution of 64 s. Periodic variability was apparent in the raw data and by folding the data from successive orbits with trial periods near that observed in the raw data, a period of  $6.75 \pm 0.03$  min was obtained. Data were also obtained with a time resolution of 48 s on January 21 and 23, 1975, when the source strength had decayed to  $0.005$  photon  $\text{cm}^{-2} \text{s}^{-1} \text{keV}^{-1}$ . The regular variability was again present with unchanged period, within the limits of the uncertainty of  $\pm 0.03$  min. In each case, the depth of modulation was large—greater than 80%.

Combining all these data allows the period to be refined to  $6.755 \pm 0.010$  min. The light curve of this periodicity is shown in Fig. 1b. The depth of modulation is  $85 \pm 3\%$ .

As the 6.755-min periodicity and large depth of modulation persisted, unchanged, within the accuracy of our measurement,

to a stage at which the source intensity had fallen below the level of the initial sighting, it is likely that this periodicity is a constant characteristic of the source and not a transient periodicity associated with the flare.

Another significant feature of this observation is the constancy of the spectral shape, both in the soft X-ray absorption and higher energy slope.

Regular variability in X-ray sources may be divided into two classes—those with periods of a few seconds or less, believed to be associated with the rotation period of a compact star, such as a neutron star, and those with periods of a few hours or more, associated with the orbital motion of a binary system. The present periodicity falls between these two groups.

The quasi-sinusoidal nature of the light curve seems to rule out an origin for the pulses from beamed radiation from rotating neutron stars, and square wave modulation, as might occur in an eclipsing binary system may also be excluded.

The light curve of the present periodicity is not unlike that of the 4.8-h periodicity of Cyg X-3<sup>4</sup>. In a model for Cyg X-3<sup>5,6</sup> the X-ray source is proposed to be a white dwarf associated, in a binary system, with a red dwarf. The X-ray modulation is the result of electron scattering in a column of material whose optical depth varies with the orbital phase of the white dwarf about its companion.



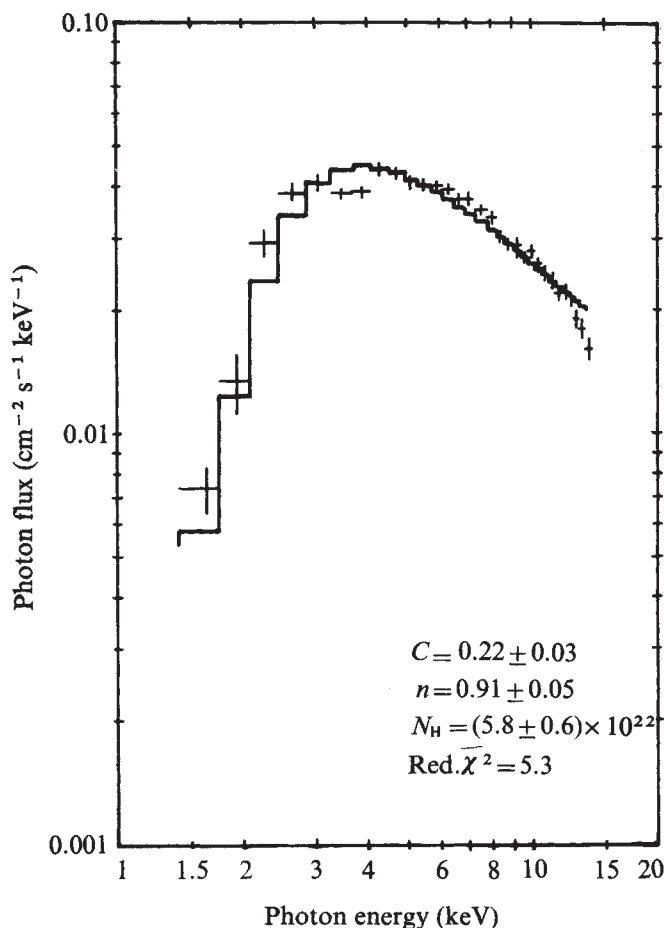


Fig. 2 Comparison between the sum of the data collected during five consecutive orbits over the period of maximum intensity and best fitting power law spectrum ( $I = C \exp(-N_H)E^{-n}$ )

If the 6.755-min period is attributable to orbital motion of a binary system, the system must be appreciably more compact than the Cyg X-3 system. In this new source, Ariel 1118-61, both objects must be compact.

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<sup>1</sup> Eyles, C. J., Skinner, G. K., Willmore, A. P., and Rosenberg, F. D., *Nature*, **254**, 577 (1975).

<sup>2</sup> Brown, R. L., and Gould, J., *Phys. Rev. D.*, **1**, 2252 (1970).

<sup>3</sup> Daltabuit, E., and Meyer, S., *Astr. Astrophys.*, **20**, 415-424 (1972).

<sup>4</sup> Sanford, P. W., *Proc. R. Soc.*, **A340**, 411-422 (1974).

<sup>5</sup> Pringle, J. E., *Nature*, **247**, 21-22 (1974).

<sup>6</sup> Davidsen, A., and Ostriker, J. P., *Astrophys. J.*, **189**, 331-338 (1974).

<sup>7</sup> Schreier, E., Levinson, R., Gursky, H., Kellogg, E., Tananbaum, H., and Giacconi, R., *Astrophys. J.*, **172**, L79-L89 (1972).

## Occurrence of diamond in a mica-garnet lherzolite xenolith from kimberlite

ALTHOUGH graphite has previously been reported from upper-mantle garnet lherzolite xenoliths from kimberlite intrusions, diamond has not previously been found in upper-mantle garnet lherzolite xenoliths from kimberlite intrusions. Diamond of primary terrestrial origin is found mainly in the rare ultramafic, potassic rock, kimberlite. Although the precise source of most diamonds in kimberlite is unknown it is generally assumed that most of the diamonds originate from

the kimberlite matrix itself. A few have, however, been found enclosed in eclogite xenoliths of widely varying composition<sup>1-3</sup> and also in rare xenoliths of garnet serpentinite from the Aykhal kimberlite diatreme in Siberia<sup>4</sup>. We report here on the occurrence of diamond in a xenolith of garnet lherzolite, the material generally believed to dominate the Earth's upper mantle on density, seismic and experimental grounds.

The xenolith (BD2125) was collected from the kimberlite of the Mothae kimberlite diatreme<sup>5</sup> in northern Lesotho. During preparation of a thin section, a small, extremely hard grain was encountered. Preliminary tests (Moh's hardness > 9, insoluble in hydrofluoric acid HF) and subsequent single-crystal X-ray diffraction proved that it was diamond. The crystal is irregular with maximum and minimum widths of 0.8 and 1.4 mm, respectively; it weighs 2.7 mg. The precession X-ray patterns show twinning on (111), but the position of the twin boundary is unknown. The surface has one near-planar face—identified as (111) by X rays—but is mostly irregular, ranging from rounded parts to irregular hackly parts, perhaps showing multiple exposures of cleavage surfaces or growth features. A prominent re-entrant at one end may result from the twinning. The diamond is essentially colourless. Surface and internal impurities occur, the latter including thin veils and two thin, flat rods about 0.01 mm long lying parallel to the flat face. One of the rods is yellow-brown and the other is colourless. Two more rods lie close to the surface, and at least one (probably several) body projects inwards from the surface.

The host xenolith is angular: 6 × 8 × 9 cm. The rock is very fresh and has a distinct fabric, with the long axes of the olivine and orthopyroxene grains showing preferred orientation parallel to the longest axis of the xenolith. In the terminology of Bouiller and Nicolas<sup>6</sup> the texture is coarse tabular. There are some triple boundaries of approximately 120° between adjacent grains, which often have curved grain boundaries; some orthopyroxenes and olivines show undulose extinction. This complex texture indicates recrystallisation towards an equilibrium texture (though this is not quite achieved), perhaps with late-stage deformation. The mode of the rock is variable, with olivine and orthopyroxene always dominant; garnet is a consistent minor mineral, but clinopyroxene is relatively rare and sporadic. The olivine and orthopyroxene grains measure up to 6 mm long, and the garnets are up to 4 mm across; the clinopyroxene grains do not exceed 3 mm in length. The garnets are surrounded by a narrow (0.2 mm) reaction rim of clinopyroxene, orthopyroxene and equant brown spinel. Three mica crystals were found in the four polished thin sections prepared for electron microprobe study. Two of them abut each other next to the reaction rim of a garnet, are about 1 × 0.5 mm in size, show light-brown pleochroism, and are multiply included. A third crystal in another thin section is similar in all respects.

Electron microprobe analyses showed uniform compositions irrespective of texture. Representative analyses are given in Table 1. The olivine (Fa<sub>7</sub>) and enstatite (Fs<sub>6</sub>) are highly magnesian, the latter containing small amounts of Al<sub>2</sub>O<sub>3</sub>, Cr<sub>2</sub>O<sub>3</sub> and CaO. The garnet is pyrope-rich with 5.5 weight% Cr<sub>2</sub>O<sub>3</sub> and little TiO<sub>2</sub>; it thus resembles the pyrope found in most common garnet lherzolites. It differs, however, from the garnets in the diamond-bearing garnet serpentinites from the Aykhal kimberlite, in which the two analysed pyropes contain 8.2 and 14.1 weight% Cr<sub>2</sub>O<sub>3</sub>, respectively. In addition, one of the Aykhal garnets contains only 1.9 weight% CaO which, although in this respect resembles some garnet inclusions in diamond<sup>3,7</sup>, serves to set it apart from the garnet in the xenolith discussed here. The clinopyroxene is a sodic diopside containing small amounts of Al<sub>2</sub>O<sub>3</sub> (1.9 weight%), Na<sub>2</sub>O (1.5 weight%), Cr<sub>2</sub>O<sub>3</sub> (1.6 weight%). The mica is a phlogopite with low TiO<sub>2</sub> (0.14 weight%) and intermediate Cr<sub>2</sub>O<sub>3</sub> (0.86 weight%). These values fall in the range for primary micas from peridotite xenoliths<sup>8</sup>. In short, the mineral chemistry is quite consistent with that of other upper-mantle, granular, mica-garnet lherzolites<sup>9</sup>.

**Table 1** Silicate analyses of the xenolith (BD 2125)

|                                | 1*    | 2     | 3     | 4     | 5      |
|--------------------------------|-------|-------|-------|-------|--------|
| SiO <sub>2</sub>               | 41.5  | 40.9  | 57.9  | 55.0  | 41.7   |
| TiO <sub>2</sub>               | 0.07  | 0.00  | 0.01  | 0.02  | 0.14   |
| Al <sub>2</sub> O <sub>3</sub> | 19.9  | 0.03  | 0.79  | 1.88  | 12.9   |
| Cr <sub>2</sub> O <sub>3</sub> | 5.50  | 0.03  | 0.33  | 1.65  | 0.86   |
| FeO                            | 5.95  | 7.00  | 4.19  | 1.80  | 2.45   |
| MnO                            | 0.32  | 0.08  | 0.11  | 0.07  | 0.01   |
| MgO                            | 20.5  | 50.7  | 36.2  | 17.8  | 26.8   |
| NiO                            | 0.01  | 0.38  | 0.10  | 0.02  | 0.25   |
| CaO                            | 5.66  | 0.03  | 0.53  | 20.4  | 0.00   |
| Na <sub>2</sub> O              | 0.02  | 0.01  | 0.10  | 1.55  | 0.06   |
| K <sub>2</sub> O               | 0.00  | 0.00  | 0.00  | 0.03  | 10.3   |
| Total                          | 99.4  | 99.2  | 100.2 | 100.2 | (95.5) |
| Si                             | 5.971 | 5.992 | 7.900 | 7.906 | 5.868  |
| Al <sup>IV</sup>               |       | 0.006 | 0.100 | 0.094 | 2.132  |
| Al <sup>VI</sup>               | 3.377 |       | 0.027 | 0.226 | 0.010  |
| Ti                             | 0.007 | 0.000 | 0.001 | 0.002 | 0.015  |
| Cr                             | 0.626 | 0.003 | 0.035 | 0.188 | 0.096  |
| Fe                             | 0.716 | 0.840 | 0.477 | 0.216 | 0.288  |
| Mn                             | 0.040 | 0.010 | 0.013 | 0.009 | 0.001  |
| Mg                             | 4.406 | 11.08 | 7.353 | 3.829 | 6.999  |
| Ni                             | 0.001 | 0.045 | 0.011 | 0.002 | 0.028  |
| Ca                             | 0.873 | 0.005 | 0.077 | 3.145 | 0.001  |
| Na                             | 0.004 | 0.003 | 0.026 | 0.433 | 0.016  |
| K                              | 0.001 | 0.000 | 0.000 | 0.005 | 1.852  |
| O                              | 24    | 24    | 24    | 24    | (22)   |

\* 1, garnet; 2, olivine; 3, orthopyroxene; 4, clinopyroxene; 5, mica. Electron microprobe analyses by Chicago 1974 11-element system. Special analyses for minor elements gave: TiO<sub>2</sub>: garnet, 0.075 weight%; olivine, 0.002 weight%; orthopyroxene, 0.014 weight%; clinopyroxene, 0.021 weight%. Cr<sub>2</sub>O<sub>3</sub>: olivine, 0.029 weight%. Na<sub>2</sub>O: garnet, 0.021 weight%.

The Ca/(Ca+Mg) ratio of diopside and the Al<sub>2</sub>O<sub>3</sub> content of enstatite provide an estimate<sup>10</sup> of temperature and pressure for pyrope-lherzolites, but the details are controversial. The Ca/(Ca+Mg) ratio of  $0.451 \pm 0.002$  for the clinopyroxene from this xenolith indicates an equilibration temperature of 1,040 °C (using the Di-En join of Davis and Boyd<sup>11</sup>) and 980 °C (using new electron microprobe data<sup>12</sup> for the Di-En join at  $3 \times 10^5$  kPa). Four analyses using a Di<sub>85</sub>Jd<sub>15</sub> standard gave Ca/(Ca+Mg) ratio of 0.449, 0.451, 0.452, 0.452.) The empirical fit of Wood and Banno (ref. 13, equation 27) to cover the substitution of the minor elements, especially Fe, Al, Cr and Ba, leads to a positive correction of about 70 °C. Preferring the direct chemical data<sup>12</sup>, however, we tentatively propose an equilibration temperature of 1,050 °C. The Al<sub>2</sub>O<sub>3</sub> content, 0.79 weight%, is too low to read from Fig. 2 of MacGregor<sup>14</sup>, and the spacing of the isopleths is nonlinear. An empirical extrapolation of experimental data by Boyd and England together with the correction for minor elements (ref. 13, equation 17) yields an estimated pressure of  $5.2 \times 10^5$  kPa; substitution of MacGregor's<sup>14</sup> data yields a value of  $4.6 \times 10^5$  kPa (B. J. Wood, personal communication). The shield geotherm of Clark and Ringwood<sup>15</sup> passes through 150 km at 1,050 °C, which is fairly consistent with the estimated pressures, and is just inside the stability field of diamond.

Apart from the intrinsic interest of this first published record of diamond in a mica-garnet lherzolite, several points should be discussed.

First, the irregular rounded shape of the diamond (apart from the flat face which may be a cleavage artefact from preparation of the thin section) is perhaps relevant to the observation<sup>16</sup> that some diamonds are a combination of two distinctive growth forms. One represents an earlier stage in which the habit is rounded or mamillary (except for limited octahedral flat faces), and the other is an overgrowth with a distinctly octahedral habit. With the proven existence of a rounded diamond in garnet lherzolite, it may be possible to speculate that during the disruption of garnet lherzolite into a kimberlite melt, small rounded diamonds act as nuclei for diamond which precipitates from the magma with a regular habit.

Second, the xenolith we have discussed contains phlogopite and the inferred pressure and temperature fall at the upper end of the range for mica-bearing lherzolites<sup>10</sup>. Two of the lherzolites described by Boyd<sup>10</sup> contain graphite as well as phlogopite, in contrast to his phlogopite-free specimens which contain no graphite and have inferred pressures and temperatures that place their equilibration within the stability field of diamond. If the phlogopite in our xenolith grew in equilibrium with the diamond, it may be inferred that the phlogopite field does extend into the stability field of diamond.

Finally, the presence of graphite in garnet lherzolite xenoliths could arise from either primary growth on the low-pressure side of the graphite-diamond curve or from the breakdown of diamond attendant on upward movement of the host lherzolite out of the diamond stability field or from a combination of both factors. The latter would occur in a manner analogous to the breakdown of pyrope in lherzolites during their rise to the Earth's surface<sup>17</sup>. This may be brought about by upwelling of an upper-mantle diapir connected with kimberlite magmatism (see ref. 18).

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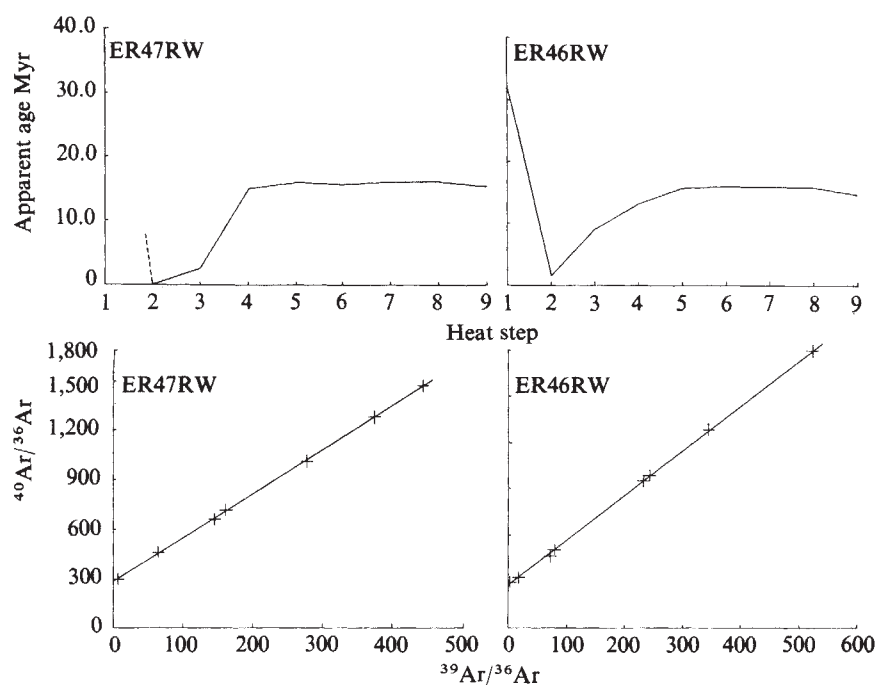
Received February 4; accepted March 7, 1975.

- Rickwood, P. C., Gurney, J. J., and White-Cooper, D. R., in *Upper Mantle Project, Spec. Publ. 2, geol. Soc. S. Afr.*, 371-393 (1969).
- Sobolev, N. V., *Australian National University Press, Publication 210* (1970).
- Reid, A. M., Brown, R. W., Dawson, J. B., Whitfield, G. G., and Siebert, J. C., *Extended Abstr. int. Conf. on Kimberlites, Cape Town*, 271 (1973).
- Sobolev, N. V., Nay, B. S., Sobolev, N. V., Lavrentyev, Yu. G., and Pospelova, L. N., *Dokl. Acad. Nauk. SSSR, Earth Sci. Sect.*, **188**, 168-170 (1969).
- Nixon, P. H., in *Lesotho kimberlites* (edit. by Nixon, P. H.), 39-47 (Lesotho Development Corporation, Maseru, 1973).
- Bouillier, A. M., and Nicolas, A., *Phys. Chem. Earth* (in the press).
- Meyer, H. O. A., and Boyd, F. R., *Geochim. cosmochim. Acta*, **36**, 1255-1273 (1972).
- Dawson, J. B., and Smith, J. V., *Nature*, **253**, 336-338 (1975).
- O'Hara, M. J., and Mercy, E. L. P., *Trans. R. Soc. Edinb.*, **65**, 251-314 (1963).
- Boyd, F. R., *Geochim. cosmochim. Acta*, **37**, 2533-46 (1973).
- Davis, B. T. C., and Boyd, F. R., *J. geophys. Res.*, **71**, 3567-3576 (1966).
- Nehru, C. E., and Wyllie, P. J., *Contr. Miner. Petrol.*, **48**, 221-228 (1974).
- Wood, B. J., and Banno, S., *Contr. Miner. Petrol.*, **42**, 109-124 (1973).
- MacGregor, I. D., *Am. Miner.*, **59**, 110-119 (1974).
- Clark, S. P., and Ringwood, A. E., *Rev. Geophys.*, **2**, 35-88 (1964).
- Seal, M., *Am. Miner.*, **50**, 105-123 (1965).
- Reid, A. M., and Dawson, J. B., *Lithos*, **5**, 115-124 (1972).
- Green, H. W., II, and Gueguen, Y., *Nature*, **249**, 617-620 (1974).

## Age of a new carbonatite locality in northern Kenya

THE ages of carbonatites which occur within the Suregei-Asille Volcanics of northern Kenya cannot be obtained at present by direct radioisotope dating, but can be derived from the <sup>40</sup>Ar/<sup>39</sup>Ar K-Ar isochron ages of intercalated felsic ignimbrites. The Suregei-Asille Volcanics crop out over an area of some 1,000 km<sup>2</sup> to the north-east of Lake Rudolf. They rest unconformably on the Precambrian basement and comprise several volcanic formations. The rock types include flood basalts; Strombolian cinder cones; aa- and pahaoehoe-flows varying in composition from ankaramitic basalt to trachyte; felsic air-fall and ash-flow deposits; lahars; carbonatite volcanics; and a variety of reworked volcanic ashes, volcanoclastic and fluvial sediments.





**Fig. 1**  $^{40}\text{Ar}/^{39}\text{Ar}$  age spectra and K-Ar isochrons obtained from samples of juvenile volcanic sanidine separated from two stratigraphically adjacent alkaline felsic ignimbrites (ER47RW and ER46RW) from the Gum Dura Formation of the Suregei-Asille Volcanics, northern Kenya. In each case the analytical data are plotted both as an age spectrum and as an isochron<sup>2,10</sup>. The shape of the age plateau obtained from ER46RW suggests the presence of a minute discrepancy caused by argon loss, but this is seen to have had a minimal effect on the isochron determination. The age plateau and K-Ar isochron from ER47RW are perfect examples of the results to be expected from completely non-discrepant dating samples.

An important marker horizon within the Suregei-Asille Volcanics is a thin welded ignimbrite of considerable extent. This distinctive felsic ignimbrite forms part of the Gum Dura Formation (R.T.W., unpublished). Within this formation, fine-grained carbonate rocks of volcanic origin are particularly important. These newly discovered carbonatites are of exceptional petrogenetic interest: first they seem to be pyroclastic rocks emplaced by ash-flow and air-fall eruptions; second, their association is with alkali-basalts and felsic ignimbrites rather than with feldspathoidal lavas and undersaturated alkaline intrusive rocks. Elsewhere in the Suregei-Asille Volcanics there are discrete volcanic centres, lava flows and pyroclastic rocks of similar carbonate mineralogy<sup>1</sup>. Recrystallisation and silicification are commonly developed in these carbonatites. The presence of thick, carbonate-rich horizons within the volcanics which surround and underlie the East Rudolf Plio-Pleistocene sedimentary basin may explain, at least in part, the frequent occurrence of calcite mineralised fault planes, calcite veining and the widespread presence of calcite cementation in the rocks of the basin.

Two ignimbrites within carbonatites of the Gum Dura Formation were sampled for dating using the  $^{40}\text{Ar}/^{39}\text{Ar}$  single sample K-Ar isochron technique<sup>2</sup>. The first sample (ER47RW) was taken from the prominent marker horizon already mentioned. The second (ER46RW) is a check sample from a less extensive ash-flow unit which underlies the first at the collection locality (ref. BH810850 on the Kenya National Grid), a river gorge on the Il Dura branch of the upper Il Eriet, some 5 km south of the Kenya-Ethiopia border. Discoloration in sample ER46RW suggests that it has been subjected to some kind of weak hydrous alteration that did not penetrate the overlying, more strongly welded horizon. Concentrates of euhedral juvenile volcanic sanidine for dating were extracted from each rock sample by crushing and hand picking using a stereoscopic polarising microscope.

The results of full  $^{40}\text{Ar}/^{39}\text{Ar}$  step-heating age analysis of the two sanidine concentrates are illustrated in Fig. 1. The results from the first dating sample (ER47RW) indicate that it contains only one age component, producing a single plateau on the age spectrum and defining a perfect K-Ar isochron of apparent age  $16.22 \pm 0.10$  Myr ( $^{40}\text{Ar}/^{36}\text{Ar}$  intercept value:  $288 \pm 4$ ). The

shape of the age spectrum obtained from the check sample (ER46RW) suggests that a slight discrepancy resulting from argon loss may be present, although the isochron plot reveals that any argon loss is virtually insignificant and a nearly perfect K-Ar isochron of apparent age  $16.14 \pm 0.13$  Myr ( $^{40}\text{Ar}/^{36}\text{Ar}$  intercept value:  $280 \pm 6$ ) was also obtained from this sample. The two K-Ar isochron ages are in agreement within the experimental limits; therefore, the marginally superior age of  $16.22 \pm 0.10$  Myr obtained for the prominent marker horizon can be accepted with confidence. Both isochrons intersect the  $^{40}\text{Ar}/^{36}\text{Ar}$  axis at a ratio of less than 295.5, indicating the presence of initial argon which had an argon isotope ratio unlike that in the modern atmosphere: thus any conventional K-Ar age determinations or calculations relating to these samples will produce discrepantly low apparent age values. The K-Ar isochron age accepted for the upper felsic ignimbrite in the Gum Dura Formation is consistent with, but considerably superior to, previous K-Ar dates from the Suregei-Asille Volcanics. In particular, it is in agreement with both the average, conventional K-Ar apparent age of  $17.3 \pm 1.4$  Myr obtained from a basalt slightly lower in the succession north of the Buluk Gap<sup>3</sup>, and with the average, conventional K-Ar apparent ages of  $14.1 \pm 1.4$  Myr,  $12.3 \pm 1.4$  Myr and  $11.6 \pm 0.5$  Myr obtained from basalts higher in the succession along the Suregei Cuesta<sup>4,5</sup>.

This precise, geologically acceptable K-Ar isochron age, confirmed from an adjacent but independent ash-flow, is an excellent example of the efficacy of the  $^{40}\text{Ar}/^{39}\text{Ar}$  single sample (or step-heating) K-Ar isochron dating technique. In addition, the generation of reproducible, perfect and near perfect isochrons from volcanic sanidines in this study makes it unlikely that complex age spectra obtained from similar analyses of the same mineral (for example, sanidine concentrates separated from pumice occurrences in the reworked tuffs of the East Rudolf sedimentary basin<sup>4,5</sup>) can be explained away or rejected as artefacts of the  $^{40}\text{Ar}/^{39}\text{Ar}$  irradiation technique.

The association between voluminous felsic volcanism and carbonatitic ash-flow volcanism in the Gum Dura Formation is unique. The firm dating of this extremely unusual episode of carbonatite magmatism at  $16.22 \pm 0.10$  Myr places it in the early Middle Miocene. At this time the Suregei-Asille region

formed part of a shallow structural depression lying between the rising Ethiopian and Kenyan basement swells<sup>6</sup>. Volcanism has been important on both swells as well as in the intervening Turkana depression since their inception. If, as seems likely, these swells and the rift valleys that eventually developed across their crests can be regarded as the surface expressions of two adjacent mantle plumes, then a detailed knowledge of the sequence and changing petrography of the magmas erupted throughout this East African Rift Province is an essential prerequisite to our understanding of these phenomena. Volcanism on the Kenya swell began with the appearance during the Lower Miocene of a number of isolated melanephelinite-phonolite central volcanoes. Further north, in the Turkana depression and in Ethiopia, extensive alkali-basalt lineage eruptions with associated ignimbrite volcanism became prevalent around this time.

Dissection of some of the large, Miocene central volcanoes in western Kenya and eastern Uganda has revealed that they possess intrusive ijolite-carbonatite cores (for example, Napak<sup>7,8</sup>). The dating of these intrusive carbonatites and their associated ijolites has proved to be particularly difficult. It has been suggested from purely geological considerations that an age within the latter part of the range 22–16 Myr may be more reasonable for the central complex of Napak than are those obtained from direct conventional K–Ar age analysis of the core rocks<sup>9</sup>. If it can be shown that carbonatite magmas of very different petrogenesis and volcanic association were available contemporaneously in East Africa during the Middle Miocene, then it may be possible to explain these differences in terms of structural setting in relation to the mantle plumes that have dominated East African geology over the past 25 Myr.

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- <sup>1</sup> Watkins, R. T., *J. geol. Soc. Lond.* (in the press).
- <sup>2</sup> Fitch, F. J., Miller, J. A., and Mitchell, J. G., in *Time and Place in Orogeny*, (edit. by Kent, P. E., et al.), (Geol. Soc. Lond., London, 1969).
- <sup>3</sup> Harris, J., and Watkins, R. T., *Nature*, **252**, 576 (1974).
- <sup>4</sup> Fitch, F. J., Findlater, I. C., Watkins, R. T., and Miller, J. A., *Nature*, **251**, 213 (1974).
- <sup>5</sup> Fitch, F. J., and Miller, J. A., in *Earliest Man and Environments in the Lake Rudolf basin: Stratigraphy, Palaeoecology, and Evolution* (edit by Coppens, Y., Howell, F. C., Isaac, G. I., and Leakey, R. E.), (University of Chicago Press, Chicago 1975).
- <sup>6</sup> Fitch, F. J., and Vondra, C. F., *ibid.*
- <sup>7</sup> King, B. C., *Mem. geol. Surv., Uganda*, **5**, 1–57 (1949).
- <sup>8</sup> Trendall, A. F., *Rep. geol. Surv. Uganda*, **12**, 1–69 (1966).
- <sup>9</sup> Bishop, W. W., Miller, J. A., and Fitch, F. J., *Am. J. Sci.*, **267**, 669 (1969).
- <sup>10</sup> Fitch, F. J., Miller, J. A., and Hooker, P. J., *J. Geol. Soc. Lond.* (in the press).

## Origin of magnesium and potassium ions in Lake Vanda, Antarctica

THE Dry Valley area of southern Victoria Land is one of the greatest ice-free areas in Antarctica and contains lakes and soils which are noted for their saline character. Particular attention has been focused on the origin of these salts, first because it contributes to an understanding of Antarctic

weathering and soil formation, second because it is related closely to the glacial history of the Dry Valley area, and third because it is connected with marine invasion and the Pecten gravels<sup>1</sup> in Wright Valley.

Suggested sources for the salts in the Dry Valley area include marine invasion of the valleys, atmospheric precipitation and rock weathering. Most published chemical studies reject marine invasion of Wright Valley because of the hydrological barrier created by the valley threshold, although Webb<sup>2</sup> presented convincing palaeoecological evidence for marine penetration of the valley. Nakai<sup>3</sup> concluded from the stable sulphur isotopic measurements of sulphates around Lake Vanda, Wright Valley that sulphates in the lake sediments of Hole No. 4 of the Dry Valley Drilling Project<sup>4</sup> and sulphate minerals around the lake originated from seawater, indicating marine invasion or atmospheric precipitation. The origin of salts in Lake Vanda has also been discussed on the basis of the distribution of evaporites and soil salts<sup>5</sup>.

Many workers have analysed six kinds of ions—Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Cl<sup>−</sup> and SO<sub>4</sub><sup>2−</sup>—in the saline lakes of the Dry Valley area. These ions are also main components of seawater. The ionic composition of the water in the lakes is, however, different from that of seawater. There are extensive deposits of thenardite, halite, sodium nitre, calcite and gypsum around Lake Vanda. Therefore, contemporary distributions of Na<sup>+</sup>, Ca<sup>2+</sup>, Cl<sup>−</sup> and SO<sub>4</sub><sup>2−</sup> are likely to bear little relation to primordial levels in the lake. On the other hand, magnesium and potassium salts are not found around Lake Vanda except in rare occurrences of epsomite and dolomite. Thus, contemporary distributions of Mg<sup>2+</sup> and K<sup>+</sup> are likely to bear some relation to primordial distribution.

Further evidence justifying selection of Mg<sup>2+</sup> and K<sup>+</sup> for study arises from the types of salts formed during the concentration of brine. The ground temperature in Wright Valley averages between −25 °C and −30 °C from March until September<sup>6</sup>. Therefore, it would be more reasonable to suppose that salts deposit as a result of fractional freezing rather than by normal evaporation. In frigid conditions, the concentration curves<sup>7</sup> for Mg<sup>2+</sup> and K<sup>+</sup> in seawater undergoing progressive cooling exhibit a fairly constant relationship between 0 and −45 °C. Values for the Mg/K ratio range from 3 to 4. There is a steady increase in concentration of both ions down to −36 °C; further lowering of temperature causes sedimentation of magnesium chloride and potassium chloride, and a reduction in concentration. These data were obtained during progressive freezing of brine samples, and the thermal history of the lake and its surroundings are probably different; in the natural situation it is probable that Mg<sup>2+</sup> and K<sup>+</sup> would remain in residual brine without the crystallisation of magnesium and potassium salts. Furthermore, if the ionic composition of primordial brine were similar to that of seawater, the Mg/K ratios would remain constant below −36 °C at which temperature the magnesium and potassium salts begin to crystallise. Therefore, it is likely that, under prevailing temperature conditions, the Mg/K ratio was relatively undisturbed during the freezing of brine in the lake.

Because of their high solubility any superficial deposits of magnesium and potassium salts on the surrounding land may be washed into the lake. From the considerations mentioned already we suggest that the Mg/K ratio of this component will also remain close to the primordial value.

Using the chemical composition data for samples of lake water collected from various parts of Lake Vanda<sup>8</sup> we classified the samples according to the values of the Mg/K ratio. Figure 1 shows the plot of the value of the Mg/K ratio against the concentration of total salts (sum of six components) in lake water. There are two types of brine in the lake water according to the values of the Mg/K ratio. The boundary value is nearly 4, which is the same as that in seawater. Since the value of the Mg/K ratio is nearly 1 in freshwater ponds in the Dry Valley area, Mg<sup>2+</sup> and K<sup>+</sup> in samples for which the ratio is less than 4 probably originate from the weathering of rock and from sea



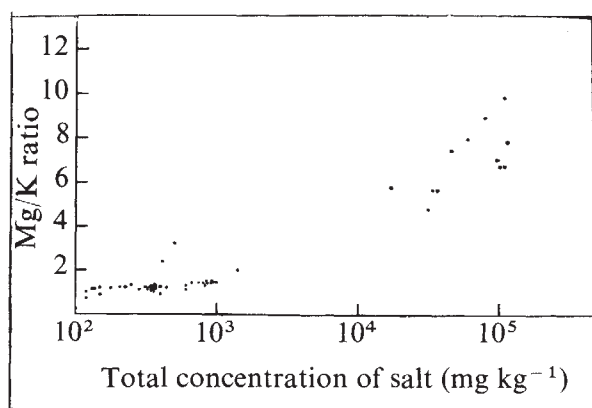


Fig. 1 The Mg/K ratio against the concentration of total salts in water of Lake Vanda.

spray. These samples exist mainly in the shallower parts of the lake, and are less dense than seawater. Rock weathering and sea spray are possible sources for salts throughout the Dry Valley area and these processes are occurring at present.

On the other hand, we suggest that  $Mg^{2+}$  and  $K^+$  in samples for which the ratio is greater than 4 originate from seawater. These samples mainly originate from parts of the lake deeper than about 45 m. In comparison with a seawater distribution, however,  $Mg^{2+}$  is enriched relative to  $K^+$ .

Although  $Mg^{2+}$  and  $K^+$  have similar concentration characteristics in a progressive cooling situation, this is unlikely to be true for all real situations in which temperature fluctuates. Nakai<sup>3</sup> concluded from the stable oxygen isotopic measurements of water in Lake Vanda that most of the present lake waters originate from a freshwater source. It is probable that in the initial stages of formation of the lake the isolated seawater evaporated to form salt deposits. Since Lake Vanda has no outlet, these salts in the Wright Valley may have been washed into the lake at persistently low temperatures when there was a paucity of liquid water. In these conditions  $Mg^{2+}$  is 3 to 4 times more soluble than  $K^+$ ; and  $MgCl_2 \cdot 12H_2O$ ,  $MgCl_2 \cdot 8H_2O$  and  $MgCl_2 \cdot 6H_2O$  are stable from  $-34^\circ C$  to  $-17^\circ C$ , from  $-17^\circ C$  to  $-3^\circ C$  and from  $-3^\circ C$  to  $117^\circ C$ , respectively. A temperature rise causes a phase change to a less heavily hydrated salt and the liberation of its own water of crystallisation in which the salt dissolves readily. On the other hand, deposited potassium chloride requires a much slower diffusion of liquid water into the region of the crystal before solution can occur. This mechanism may help to explain the enrichment of  $Mg^{2+}$  relative to  $K^+$  during the accumulation of freshwater in the lake.

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<sup>1</sup> Nichols, R. L., *Research in the Antarctic*, (edit. by Quam, L. O.) 293, (American Association for the Advancement of Science, Washington, DC, Publication 93, 1971).

<sup>2</sup> Webb, P. N., *Antarctic J. U.S.*, 7 (6), 226 (1972).

<sup>3</sup> Nakai, N., *Bulletin of the Dry Valley Drilling Project* (Northern Illinois University), 4, 46 (1974).

<sup>4</sup> Cartwright, K., Treves, S. B., and Torii, T., *ibid.*, 3, 49 (1974).

<sup>5</sup> Morikawa, H., and Ossaka, J., *ibid.*, 4, 39 (1974).

<sup>6</sup> Thompson, D. C., Bromley, A. M., and Craig, R. M. F., *N. Z. J. Geol. Geophys.*, 14, 477 (1971).

<sup>7</sup> Thompson, T. G., and Nelson, K. H., *Am. J. Sci.*, 254, 227 (1956).

<sup>8</sup> Yamagata, N., Torii, T., and Murata, S., *Antarctic Rec.*, 29, 2339 (1967).

## Measurement of the frequency of the methane-stabilised laser at 3.39 $\mu m$ and of the R(32) transition of $CO_2$ at 10.17 $\mu m$

ACCURATE, infrared, frequency and wavelength measurements now constitute the best means of determining the speed of light, and provide a link between the standards of length and time. We measured the frequency of the methane-stabilised He-Ne laser at 3.39  $\mu m$ , and found good agreement with the result obtained by Evenson *et al.*<sup>1</sup>. The methane frequency was related to the caesium standard by using both the R(12) transition of  $CO_2$  at 9.3  $\mu m$ , which has already been measured<sup>2</sup>, and the R(32) transition of  $CO_2$  at 10.17  $\mu m$ .

Our result for the  $F_2(2)$  component<sup>3</sup> of the P(7) transition of the  $v_3$  band of methane is

$$f_{CH_4} = 88,376,181,610 \pm 70 \text{ kHz},$$

where  $f_{CH_4}$  and its uncertainty are rounded to the nearest 10 kHz.

The frequency which we obtained for the  $CO_2$  R(32) transition at 10.17  $\mu m$  is

$$f_{R(32)} = 29,477,160,862 \pm 21 \text{ kHz}.$$

The uncertainties consist of the root sum of squares of the random and systematic components which are, respectively, ( $\pm$  parts in  $10^{10}$ ) 2.1 and 7.4 for  $f_{CH_4}$  and 1.8 and 6.8 for  $f_{R(32)}$ .

The laser frequencies were measured by using the harmonic mixing properties of metal-insulator-metal point contact diodes (see ref. 2). The frequency measurements were carried out in two stages:

$$f'_{R(32)} = f'_{R(12)} - 3f_{HCN} - 27 \text{ GHz} + \Delta f_{beat} \quad (1)$$

$$f_{3.39} = 3f'_{R(32)} - 55 \text{ GHz} + \Delta f_{beat}; \quad (2)$$

where  $f_{3.39} = f'_{CH_4} - 50 \text{ MHz}$

The primes indicate that the frequencies are those of stabilised lasers rather than those of the reference transitions. The parameter  $f_{HCN}$  is the frequency of an 891 GHz, HCN laser<sup>2</sup>, and  $f_{3.39}$  is that of a 40 mW, single mode He-Ne laser (offset locked to the methane-stabilised laser) introduced to provide sufficient power for harmonic mixing.

In stage (1) 39 blocks of 10 readings were obtained on three days over a 12 week period. The standard error of the mean,  $\sigma_m$ , of the 39 averages was 1.3 parts in  $10^{10}$ . In stage (2) about 60 readings per day were obtained on five days over a three week period. For each day's results,  $\sigma_m$  was approximately 1.1 parts in  $10^{10}$  and, because of the larger uncertainty of the correction applied to each daily mean for the background slope, there was no further improvement in the random uncertainty. (In each of the two stages the standard deviation of a single reading was about 1 part in  $10^9$  and the day-to-day variation in the mean corrected result was about  $\pm 3$  parts in  $10^{10}$ .) The total random uncertainties (parts in  $10^{10}$ ) for our  $f_{R(32)}$  and  $f_{CH_4}$  results, 1.8 and 2.1, respectively, arise from combining 1.3 from the base  $CO_2$  R(12) measurement<sup>2</sup> with 1.3 from stage (1) for  $f_{R(32)}$  and with the further 1.1 from stage (2) for  $f_{CH_4}$ .

The two  $CO_2$  lasers were each stabilised by controlling the frequency with reference to the saturated-absorption dip observed using the 4.3  $\mu m$  fluorescence<sup>1</sup> from an external  $CO_2$  gas cell. Details of these stabilised lasers will be published.

**Table 1** Systematic uncertainties ( $\pm$  parts in  $10^{10}$ )

| Source                                              | Contributions<br>to $f_{R(32)}$ and to $f_{CH_4}$ |     |
|-----------------------------------------------------|---------------------------------------------------|-----|
| Rubidium standard against caesium                   | 0.3                                               |     |
| Counting errors                                     | 2                                                 |     |
| Room temperature drift                              | 2                                                 |     |
| HCN laser asymmetry*                                | 2                                                 |     |
| CO <sub>2</sub> R(12) laser reproducibility         | 3                                                 |     |
| CO <sub>2</sub> R(12) transfer (quadrature sum)†    | 4.6                                               |     |
| CO <sub>2</sub> R(32) laser reproducibility         | 3                                                 | 3   |
| CO <sub>2</sub> R(32) offset from transition centre | 4                                                 |     |
| CH <sub>4</sub> stabilised laser reproducibility    |                                                   | 4   |
| CH <sub>4</sub> offset from transition centre       |                                                   | 3   |
| Quadrature sum                                      | 6.8                                               | 7.4 |

\*An allowance for any difference between the frequency measured by time-averaged counting and that obtained by observing a spectrum analyser display.

†The CO<sub>2</sub> R(12) transfer sum combined with a further 4 parts in  $10^{10}$  to allow for offset from the transition centre gives our present estimate of systematic uncertainty (6.1 parts in  $10^{10}$  against the previous 8.3) for the CO<sub>2</sub> R(12) measurement (see ref. 2). (The allowances for HCN laser asymmetry and CO<sub>2</sub> laser reproducibility have been reduced as a result of further experimental investigation.)

The methane-stabilised He-Ne laser<sup>5</sup> had a d.c.-excited gain tube which produced maximum power output at a frequency 100 MHz lower than the methane saturated-absorption feature. This was a small peak in the output power produced by an intracavity absorption cell 100 mm long, filled to 40 mTorr. This cell resulted in a feature only 0.2% as high as the maximum power output. The first-derivative technique used for locking necessitated a correction of about 60 kHz (6.8 parts in  $10^{10}$ ) for the background slope. The main contributions to systematic uncertainty, including those now considered appropriate to our previous CO<sub>2</sub> R(12) result, are summarised in Table 1.

This measurement of the methane frequency agrees well with that of Evenson *et al.* (88,376,181,627  $\pm$  50 kHz). It is of interest that their value, together with four independent measurements of the wavelength, was used in June 1973 by the Comité Consultatif pour la Définition du Mètre to arrive at a recommended value for the speed of light<sup>6</sup> with an uncertainty of  $\pm 4$  parts in  $10^9$ . Although this uncertainty stems mainly from the determinations of wavelength, and is much larger than the quoted uncertainty of the National Bureau of Standards (NBS) frequency measurements, the good agreement of our result with theirs reduces the chance that some unsuspected error remains, the more so because our experimental method differed considerably in detail, though not in broad principle, from that of the NBS group.

The systematic uncertainties associated with the methane-stabilised laser are much reduced in an improved type now being tested, and further frequency measurements will be made. These results were communicated (with a provisional assessment of the uncertainties) to the Conference on Precision Electromagnetic Measurements, in London on July 4, 1974.

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<sup>1</sup> Evenson, K. M., Wells, J. S., Petersen, F. R., Danielson, B. L., and Day, G. W. *Appl. Phys. Lett.*, **22**, 192–195 (1973).

<sup>2</sup> Blaney, T. G., Bradley, C. C., Edwards, G. J., Knight, D. J. E., Woods, P. T., and Jolliffe, B. W., *Nature*, **244**, 504 (1973).

<sup>3</sup> Hougen, J., *J. chem. Phys.*, **39**, 358–363 (1963).

<sup>4</sup> Freed, C., and Javan, A., *Appl. Phys. Lett.*, **17**, 53–56 (1970).

<sup>5</sup> Barger, R. L., and Hall, J. L., *Phys. Rev. Lett.*, **22**, 4–8 (1969).

<sup>6</sup> Terrien, J., *Metrologia*, **10**, 9 (1974).

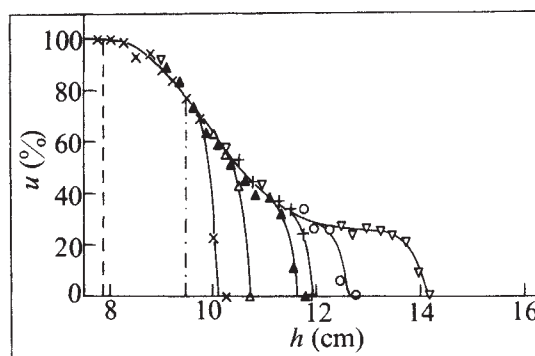
## Capillary rise in porous media

USUALLY the porespace in a porous medium is visualised as a more or less complicated assembly of isolated or interconnected capillaries, and such porespace models are used in describing transport phenomena in porous media. If capillary rise of a (wetting) liquid in a homogeneous irregular packing of rotund particles is considered, however, it seems that none of the existing porespace models is capable of explaining, not even qualitatively, the observed phenomena<sup>1</sup>.

This may be illustrated by presenting two experiments; the capillary rise of glycol and of *n*-heptane, in a homogeneous packing of polystyrene spheres. When equilibrium has been reached, it can be observed that for glycol there is a sharp front between the completely saturated part of the porous medium and the dry part of the porous medium; that is, no saturation gradient is displayed by the system glycol-polystyrene. In the case of heptane a wide saturation gradient is observed in the equilibrium situation (Fig. 1). Within the conventional porespace models the former result suggests a model of equally sized cylindrical capillaries, whereas the latter result suggests a model of capillaries of varying size. It can therefore be seen that these two results lead to inconsistent pictures of what is physically the same packing.

Numerous experiments show that the contact angle,  $\theta$ , seemingly has some bearing on this anomaly. The contact angle is a notorious parameter to determine for flat smooth surfaces, and for particles (such as beads or sand) a direct determination is almost impossible. From the work of Zisman<sup>2</sup> and others it may be concluded that  $\theta = 0^\circ$  for heptane-polystyrene and  $25^\circ$ – $50^\circ$  for glycol-polystyrene. Capillary rise of water in sand yields a wide saturation gradient at equilibrium when the sand has been cleaned by heat treatment. No saturation gradient (and a lower capillary rise) is observed when the sand is slightly contaminated. Apparently, a wide saturation gradient corresponds to  $\theta$  being approximately zero and the absence of a saturation gradient corresponds to  $\theta$  being above some critical value.

We can explain the correlation between  $\theta$  and the width of the saturation gradient for irregular sphere packings. The basic



**Fig. 1** Capillary rise of *n*-heptane in a homogeneous packing of polystyrene spheres (particle size 175–250  $\mu$ m);  $h$  is the height above the free liquid surface and  $u$  is the volume per cent porespace filled with liquid. The results are from two different experiments with two different packings. The exact form of the saturation gradient at equilibrium is somewhat uncertain because, at the times involved (10–40 d), secondary phenomena (capillary condensation, temperature fluctuations) become significant. The homogeneity of the packing and the liquid saturation is measured using an X-ray absorption technique<sup>3</sup>. The absence of a saturation gradient, as for glycol, means, operationally, that the saturation gradient is smaller than 1 mm. First experiment after:  $\times$ , 4 h;  $\blacktriangle$ , 40 h. Second experiment after: — — —, 0.5 h; — — — — —, 2.5 h;  $\triangle$ , 5 h; +, 50 h;  $\circ$ , 122 h;  $\nabla$ , 480 h.



reason why all porespace models fail is that they entail the presence of a large number of isolated menisci in the 'pores' of the porous medium. This is not the case. During capillary rise the liquid front consists of one continuous meniscus. From this it necessarily follows that the meniscus consists of concave parts separated by anticlastic parts.

Anticlastic means that the two main radii of curvature are of opposite sign. We shall use the term 'meniscus' as an abbreviation for the 'concave or anticlastic part of the liquid-vapour interphase'.

The anticlastic parts or  $\alpha$  menisci lie between the adjacent parts of two spheres. The concave parts or  $\gamma$  menisci are contained by three or more spheres and three or more  $\alpha$  menisci. The mechanism of capillary rise is governed by a continuous merging and generation of  $\alpha$  and  $\gamma$  menisci in the porespace, consisting of 'cavities' connected by 'windows'. Continuity of transport is sustained by two intersupporting mechanisms. First, the filling of windows by merging  $\alpha$  menisci (the closure of the window generates new  $\alpha$  menisci. Second, the filling of cavities by merging  $\gamma$  menisci (the closure of cavities generates new  $\alpha$  and  $\gamma$  menisci). Primarily, capillary rise is dependent on the first mechanism—that is, both  $\alpha$  and  $\gamma$  menisci contribute to the advancement of the liquid front, but the  $\alpha$  menisci are the leading agents. The merging  $\alpha$  menisci in a window may generate enough new  $\alpha$  menisci to maintain continuity of transport by  $\alpha$  menisci only. The  $\gamma$  menisci are necessary to fill the cavities but they cannot, by themselves alone, maintain continuity of transport, because, on average, the number of  $\gamma$  menisci necessary to close a cavity is larger than the number of new  $\gamma$  menisci generated by the closing cavity.

The maximum possible amount of capillary rise is determined by the curvature at which two  $\alpha$  menisci merge in the smallest window of the packing, that is in any window between three contiguous spheres. For  $\theta = 0$  this curvature is given by  $Cd = 0.11$ , where  $C$  is the sum of the inverse radii of curvature and  $d$  is the particle diameter<sup>4</sup>.

The curvature calculated from the observed maximum capillary rise (that is, the top of the saturation gradient, about 14 cm in Fig. 1) of various liquids in glass beads and polystyrene spheres yielded values of  $Cd$  in the range 0.10–0.12.

As a first approximation the saturation gradient at equilibrium after capillary rise is a function of part of the window-size distribution. In an irregular sphere packing there are a large number of smallest windows<sup>3</sup>, so it is possible that no saturation gradient is observed when the  $\alpha$  menisci filling the smallest windows can maintain continuity of transport, together with the  $\gamma$  menisci. Quantitative data on the curvature of  $\alpha$  and  $\gamma$  menisci and the statistical properties of particle packings are required to predict the width of the saturation gradient. Even for the most simple cases these values are not—or are hardly—known. It is known, however, that with increasing  $\theta$  the curvature of the  $\alpha$  menisci decreases more rapidly than that of the  $\gamma$  menisci<sup>4,6</sup>. This implies that at higher values of  $\theta$  the contribution of the  $\gamma$  menisci becomes more important, and thus that less  $\alpha$  menisci are necessary to maintain continuity of transport. Therefore, the hypothesis is put forward that the width of the saturation gradient decreases with increasing  $\theta$ . Above some critical value of  $\theta$  the maximum capillary rise is determined solely by the filling of the smallest windows by merging  $\alpha$  menisci, and thus no saturation gradient is observed.

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<sup>1</sup> Brakel, J. van, *Powder Technol.*, **11**, 205–236 (1975).

<sup>2</sup> Ellison, A. H., and Zisman, W. A., *J. phys. Chem.*, **58**, 503–506 (1954).

<sup>3</sup> Scott, G. D., *Nature*, **194**, 956–958 (1962).

<sup>4</sup> Melrose, J. C., and Wallick, G. C., *J. Phys. Chem.*, **71**, 3676–3678 (1967).

<sup>5</sup> Brakel, J. van, and Heertjes, P. M., *Powder Technol.*, **9**, 263–271 (1974).

<sup>6</sup> Princen, H. H., *J. Colloid Interface Sci.*, **10**, 359–371 (1969).

## The modulation contrast microscope

THE 'modulation contrast microscope' is a new type of imaging system with which transparent or phase objects can be visualised with a clarity and contrast equal to, or greater than, that of other light microscope systems. A bright field microscope is readily converted into a modulation contrast microscope by the addition of an aperture slit before the condenser and a modulator after the objective in a plane conjugate to the slit, the Fourier plane (Fig. 1). The modulation contrast microscope makes visible phase gradients, optical gradients and surface slopes.

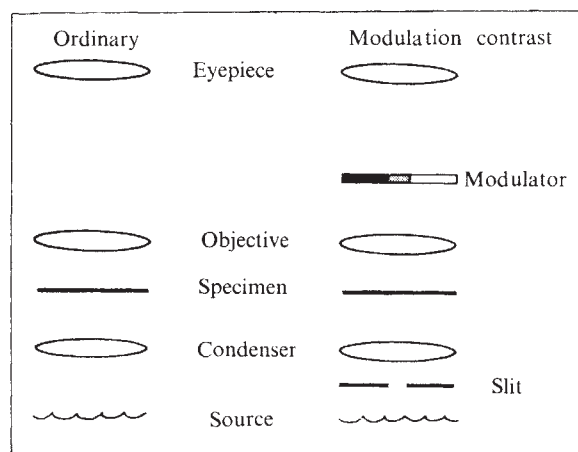


Fig. 1 Modulation contrast microscope schematic compared with bright field microscope.

Images viewed under modulation contrast are unobscured by phase objects above or below the plane of focus (optical sectioning). A transparent specimen, the human epithelial cheek cell, when viewed under a  $\times 40$  objective, NA 0.65 (Fig. 2a), reveals nucleus and cytoplasmic granules and cell edges as a three-dimensional image. Focused at another level, the cell membrane's corrugated folds and adhering bacteria are resolved (Fig. 2b).

Modulation contrast is most sensitive to gradients normal to the slit orientation; the system is directional, as indicated by the direction of the optical shadowing at the boundaries of the nucleus in Fig. 2a. The image is shadowed in the direction of gradient detection; bright on one side and dark on the other.

The resolution of the modulation contrast microscope is shown to be comparable to bright field and interference contrast. A test object for the  $\times 40$  objective is the diatom, *Pleurosigma angulatum*, the pore spacing of which is at its resolution limit. These pores are clearly delineated by modulation contrast.

The principles underlying the design of the modulation contrast microscope may be explained by describing the operation of the modulator, a specially designed filter in the Fourier plane. An elementary modulator (Fig. 3a) has three zones of different transmittances such that there is a dark region,  $T_D$ , a grey region,  $T_G$ , and a bright region,  $T_B$ , with values such that

$$T_B > T_G > T_D$$

For most objects, transmittance values approximating  $T_B = 100\%$ ,  $T_G = 15\%$  and  $T_D = 1\%$  provide satisfactory images of a wide range of gradients. The modulator is placed in the Fourier plane beyond the objective where the luminous image

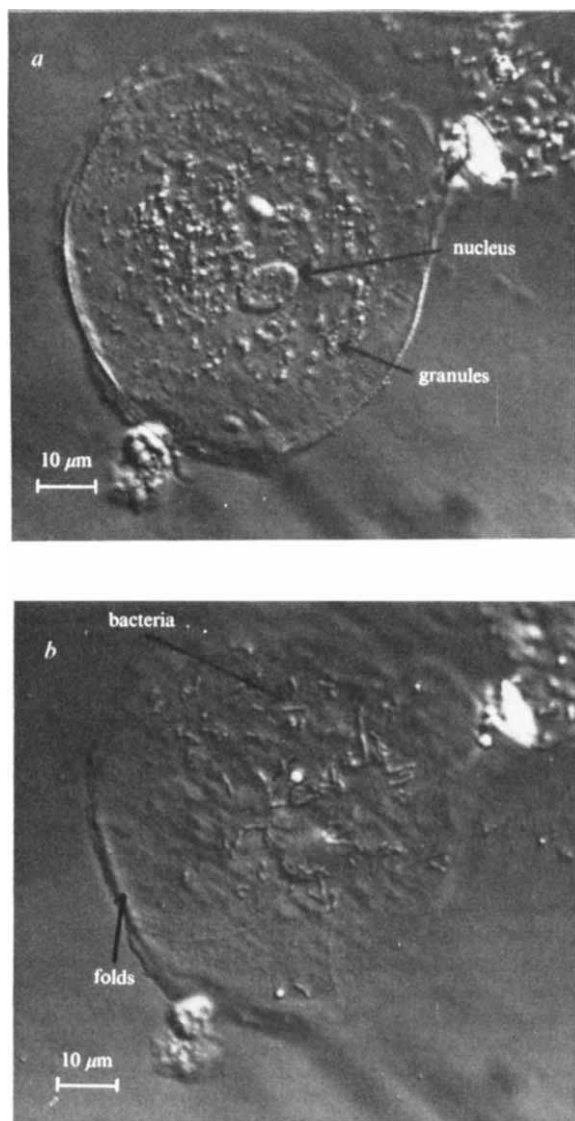


Fig. 2 Human epithelial (cheek) cells seen under modulation contrast with  $\times 40$  objective, demonstrating optical sectioning. *a*, Nucleus and cytoplasmic granules seen; *b*, cell surface without obscuring by lower structures; cell membrane folds and adhering bacteria seen.

of the slit falls on and coincides with the grey region of the modulator. A schematic diagram (Fig. 4) shows that light passing through the specimen where there are no gradients must pass through the grey region of the modulator where it is reduced to 15% of its former intensity and proceeds to the image plane, producing, for the most part, a grey background illumination. Light passing through positive gradients is refracted, in whole or in part, into the bright region, producing brighter features in the microscopic image; negative slopes direct rays which are attenuated by the dark region to produce darker features in the image. This type of modulation leads to an illusion of three-dimensionality which does not represent the geometric object, but the variation in optical density. The modulator affects only light amplitude and does not cause phase changes. Measurements on a Michelson interferometer confirmed phase changes, if any, as below  $\lambda/20$ .

Similarly, the three zones may be regions with different colours, but with transmittance ratios as in a neutral density modulator. Each optical gradient then produces intensities

as before, but in colour; similar gradients will have similar colours.

Resolution is dependent on the exit pupil of the Fourier plane which is approximately the bright region of the modulator. In a symmetrical system, where the grey region is on the optic axis, the resolution approaches  $\lambda/NA_{obj}$ , equivalent to axial illumination as described by Abbe. To maximise resolution, the grey region is offset to the edge of the exit pupil; then the dark side of the modulator is outside the exit pupil (Fig. 3*b*). To match the offset modulator, the aperture slit is also offset. In these conditions, resolution approaches  $\lambda/2NA_{obj}$ , that of oblique illumination, and is maximised.

An objective containing a modulator can be used successfully for bright field observation of stained specimens by removing the slit aperture.

The theory underlying modulation contrast microscopy shows that phase gradients in the object plane distribute zero order amplitude across the entire exit pupil. The modulator preserves the sign of the gradient, processing differently the zero orders of opposite gradients, and the image contrast and visibility is relative to background intensity, set, primarily by grey region modulator transmittance.

A plane wave entering the system in complex notation, is  $e^{-ikz}$  where  $k$ , the wave constant in air, is  $2\pi/\lambda$  and  $z$  is the direction of the optic axis. The wave constant for a ray passing through the object is then  $k' = (n_o - n_m)k$  and  $z = x \tan \alpha$  where  $\alpha$  is the gradient. Let  $\phi = k'\alpha$ , then  $e^{-i\phi x}$  describes the wave leaving the gradient. The object function in the Fourier integral is then  $e^{-i\phi x}$  and the maximum energy for different gradients is distributed across the Fourier plane.

When  $e^{-i\phi x}$  represents the gradient in any phase object, then the amplitude function in the Fourier plane is

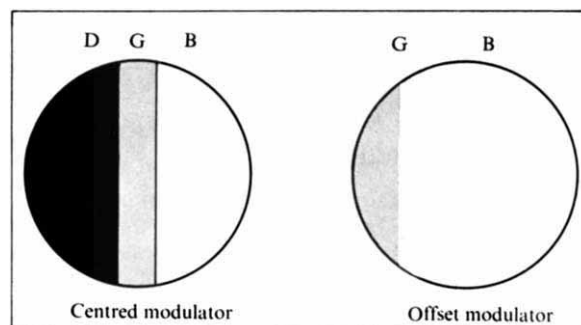
$$U(\theta) \sim \int e^{-i\phi x} e^{-i\theta x} dx \quad (1)$$

where  $\theta$  is the angular dimension in the Fourier plane and  $\phi$  represents the phase gradient in the object and  $x$  is the dimension in the object plane. The amplitude distribution across the Fourier plane is

$$U(\theta) \sim \sin(\theta \pm \phi) / (\theta \pm \phi) \quad (2)$$

For simplicity, the width of the object has been omitted. The maximum amplitude in the Fourier plane for any phase gradient in the object is not at zero angular position, but at  $\theta = \pm\phi$ . In the absence of phase gradients, the maximum amplitude would be located at  $\theta = 0$ , or at the conjugate of the source aperture. In practice, the offset modulator is used. Then, for an incident angle of illumination,  $\gamma$ ,  $\phi$  is replaced by  $\gamma \pm \phi$ . For gradients with  $\phi \neq 0$ , the zero order amplitude is shifted out of the grey region and is modulated above or below this

Fig. 3 Elementary modulator design with three transmittance regions within exit pupil. *a*, with centred grey region; *b*, with offset grey region to maximise resolution.





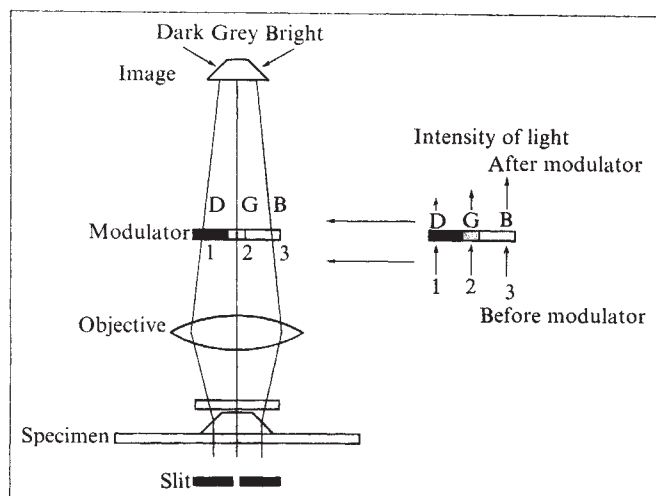


Fig. 4 Schematic diagram indicating the regions of the modulator which process light from the phase gradients in the object to form an image of contrast. No phase changes are introduced by the modulator.

value by modulator action, producing image contrast. This type of modulation leads to a three-dimensional appearance in the image plane.

Our experimental results are consistent with equation (2). Phase gradients shift the maximum intensity peak (zero order) of the diffraction pattern of a specimen feature away from the grey region and distribute the zero order (as well as higher orders) throughout the Fourier plane. It has been said that the zero order of the pattern carries no information<sup>1</sup>. We have shown this to be untrue. The location of the zero order in the Fourier plane is a measure of the optical gradient. Its intensity, with or without modulator processing, controls the intensity or contrast in the final image; without a modulator, there is no contrast between the gradients and the background. Abbe<sup>2</sup> developed the diffraction theory of image formation for amplitude objects; Zernike<sup>3</sup> extended the theory to phase objects. Dodd<sup>4</sup> discusses his schlieren microscope, stating that gradients shift zero orders. We have carried the imaging theory further by preserving the sign of phase gradients while maintaining full resolution; the theory is described in detail elsewhere<sup>5</sup>.

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<sup>1</sup> Lipson, G., and Lipson, H., *Optical Phys.*, 271, (Cambridge Univ. Press, Cambridge 1969).

<sup>2</sup> Abbe, E., *Archiv für Mikr. Anat.*, 9, 413 (1873).

<sup>3</sup> Zernike, F., *Physica*, 9, 686 and 674 (1942).

<sup>4</sup> Dodd, J. G., *Modern Microscopy*, (McCrone Microscope Publications, Chicago, in the press).

<sup>5</sup> Hoffman, R., and Gross, L., *Appl. Optics*, (in the press).

## Early domesticated sorghum from Central Sudan

LARGE quantities of carbonised *Sorghum bicolor* (L.) Moench grains, spikelets (Fig. 1a) and inflorescence fragments sorted from about 2 foot<sup>3</sup> of charred material found in a storage pit at Jebel et Tomat (13° 36'N, 32° 34'E) in Central Sudan (Fig. 2), and small amounts of carbonised sorghum found in eleven levels of the midden excavated there, suggest that sorghum

was the staple grain of people who inhabited the site. The date of 245±60 AD (UCLA 1874M) was obtained from a concentration of carbonised plant remains in the floor of the pit, which was dug into the dark clay loam on which the midden rests (Fig. 3) probably at about the same time as the accumulation of the middle or beginning of the upper unit of the midden. The remains of wickerwork matting and many fragments of thick stalks of cereal grass suggest that the pit may have been a silo lined with stalks and mats. If so, it is not dissimilar to the pits made today in the area for storing grain.

Most of the carbonised sorghum grains examined had 'popped' or become distorted in shape in the process of carbonisation, but sufficient specimens were well enough preserved to indicate the approximate size and shape of the grain. Undistorted carbonised grains were 3–3.4 mm long and 2.3–2.9 mm wide (Fig. 1b).

Jebel et Tomat is an inselberg of granite, gneiss and basic igneous rocks. Radiocarbon dates of settlement debris excavated on the Jebel indicate the site was occupied from about 40 BC to 430 AD. Charcoals from the excavations identified by the Jodrell Laboratory, Kew, as *Acacia* sp., *Salvadora persica*, *Ziziphus* sp. (probably *Z. spina-christi*), and *Ficus* sp., are evidence that the region was about as arid during the period of midden accumulation as it is today (G. Wickens, personal communication). The abundance of opal phytoliths in the midden further indicates that there was then no scarcity of grass and reeds in the immediate vicinity of the site<sup>1</sup>.

The settlement debris suggests a sizeable population, because it covers more than 12 acres and because 80–120 cm, on average, is the depth of the occupation midden, which consists of a grey-brown sandy loam, stony in the upper levels and more compact in the lower but lacking any clear evidence of disconformity. Scattered through the midden were potsherds, grindstone fragments, flaked stone debris and tools, pottery labrets and anthropomorphic and zoomorphic figurines of baked clay, together with bones from food waste, shells, charcoals and fragmentary plant remains. The prehistoric population seems to have been negroid, similar to that from Jebel Moya<sup>2</sup> and, although they were still using stone for cutting equipment and ground axes, a trace of iron is present, together with beads of blue faience and zeolite that were probably obtained through trade.

On the basis of colour, consistency and small changes in lithology, the deposit has been divided into three units the accumulation of which could have followed brief periods when the site may have been abandoned (Fig. 4). Pits for storage and other uses as well as graves dug by the later occupants into the earlier beds have been the cause of no small amount of disturbance. A run of five radiocarbon dates (Table 1) was obtained and these reflect this disturbance since the three lower ones are reversed. The dates obtained, however, confirm the evidence of the cultural remains (pottery and stone tools) that the accumulation represents a single, continuing occupation of not particularly long duration at the beginning of the first millennium AD and so broadly contemporary with Meroitic civilisation in Nubia. The well known site of Jebel Moya, some 60 km to the east and excavated in 1910–14 by Sir Henry Wellcome<sup>3</sup>, is believed to be in part contemporary with Jebel et Tomat since the pottery traditions from both are similar.

The economy at both Jebel et Tomat and Jebel Moya was one of mixed farming. At Jebel et Tomat most of the meat was obtained from domestic cattle and sheep/goats, supplemented by fishing, fowling and the hunting of gazelle and small antelope; *Pila* shells were also collected, either for food or bait.

Examination of the carbonised plant material led to the following conclusions:

(1) The sorghum from the storage pit was fully domesticated, domestication being indicated by the loss of anatomical features which facilitate dispersal of seed by natural agents. Spikelets bearing grain in the storage pit were still attached to branchlets of the inflorescence or rachis fragments. If the sorghum were not domesticated, the spikelets bearing the grain

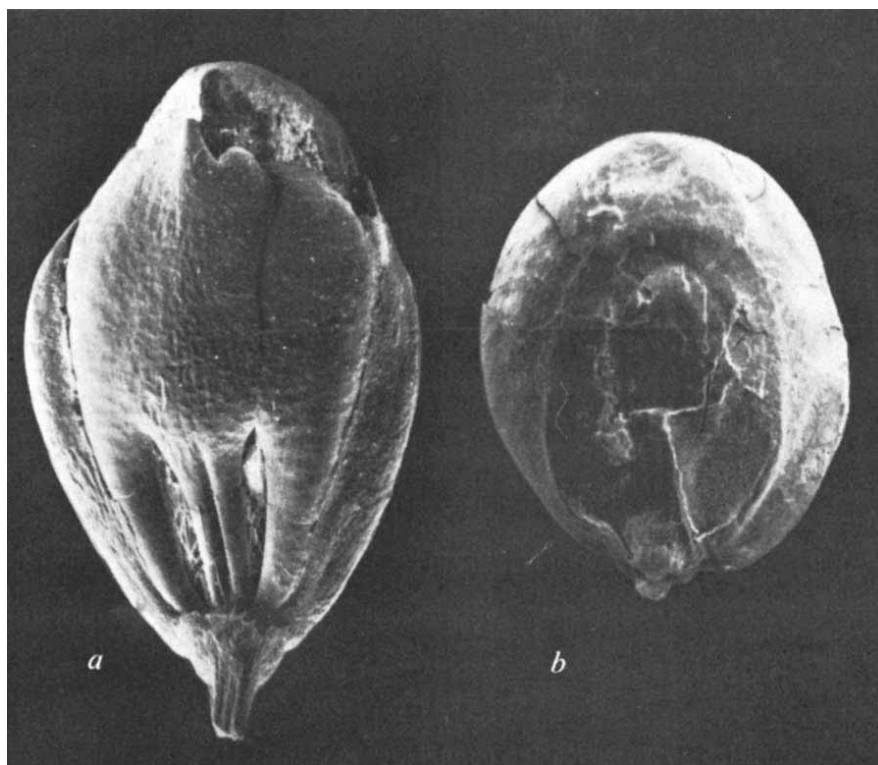


Fig. 1 *a*, Electron micrograph ( $\times 12$ ) of spikelet of *Sorghum bicolor* (L.) Moench race bicolor, from the storage pit excavated at Jebel et Tomat. The top of the grain expanded in the process of carbonisation. Attachment of branchlet of the inflorescence indicates that this is domesticated sorghum. Photo: J. N. Brunken, *b*, Electron micrograph ( $\times 12$ ) of a carbonised grain of *Sorghum bicolor* race bicolor from the Jebel et Tomat storage pit. This view shows the embryo. Photo: J. N. Brunken.

would have been separated from the supporting branchlets by formation of a zone of abscission at the base of the glumes which facilitates dispersal of seed.

(2) The sorghum from the storage pit belongs to the race bicolor of *Sorghum bicolor*. This is indicated by the fact that the grain is fully enclosed by the glumes<sup>4</sup>. The size and shape of the carbonised grain and spikelets are well within the range of variation of cultivated sorghum of race bicolor grown in many parts of Africa at present.

(3) There is no readily apparent difference in the shape and size of sorghum grains found in various levels of the midden excavated at Jebel et Tomat when compared with sorghum from the storage pit.

(4) Sorghum grain preserved in the storage pit and midden is smaller and more 'primitive' in morphology than that grown in the area at the present. There are several possible explanations for the apparent difference.

First *Sorghum bicolor* race bicolor preserved in the storage pit at Jebel et Tomat may have been only one of several kinds of sorghum grown in the area 1,700–1,900 yr ago. Larger-grained sorghum may have been stored in other pits which simply were not preserved.

Second *Sorghum bicolor* race bicolor may represent the only kind of sorghum grown at Jebel et Tomat but may not be an indication of how much progress had been made in selection of highly derived sorghums in other parts of Africa by  $245 \pm 60$  AD.

Third sorghum from Jebel et Tomat may indicate the progress made by 1700 BP in the course of selection of highly derived sorghums now grown in the north-eastern part of the African savanna. If so, the selection and change resulting in the greater size and the morphological characteristics of sorghums now typical of much of Central and southern Sudan may have occurred in the last 1,700 yr.

Table 1 Radiocarbon dates from the prehistoric settlements at Jebel et Tomat and Jebel Moya

| Sample     | Jebel et Tomat excavation       | Location                                       | depth (cm) | <sup>14</sup> C date (yr b.p.) | AD/BC date        |
|------------|---------------------------------|------------------------------------------------|------------|--------------------------------|-------------------|
| UCLA1864   | T.T.1                           | Upper unit A/1                                 | 40–50      | $1,600 \pm 80$                 | $350 \pm 80$ AD   |
| UCLA1874I  | T.T.1                           | Middle unit A/0                                | 60–70      | $1,930 \pm 60$                 | $20 \pm 60$ AD    |
| UCLA1874J  | T.T.1                           | Middle unit A/1                                | 70–80      | $1,770 \pm 60$                 | $180 \pm 60$ AD   |
| UCLA1874K  | T.T.1                           | Middle unit A/1                                | 80–90      | $1,735 \pm 60$                 | $215 \pm 60$ AD   |
| UCLA1874M  | T.T.1                           | Upper/Middle Unit A/O (Storage pit)            | 110–120    | $1,705 \pm 60$                 | $245 \pm 60$ AD   |
| SUA-67     | Soil pit on periphery of midden | Dark clay loam                                 | 40–80      | $4,540 \pm 200$                | $2,590 \pm 20$ BC |
| Jebel Moya |                                 |                                                |            |                                |                   |
| UCLA1874D  | Test pit: western perimeter     | Compact light brown gritty sandy loam (unit 3) | 80–90      | $4,200 \pm 80$                 | $2,250 \pm 80$ BC |
| UCLA1874E  | Test pit: western perimeter     | Compact light brown gritty sandy loam (unit 3) | 90–100     | $4,200 \pm 80$                 | $2,250 \pm 80$ BC |

The above dates are on charcoal excepting SUA-67 which is on shell.



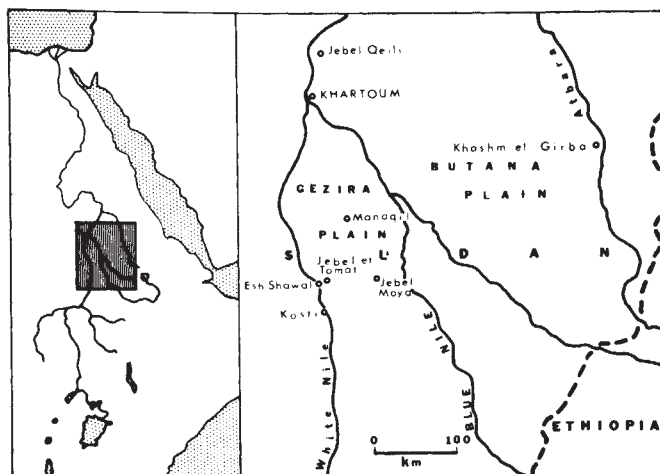
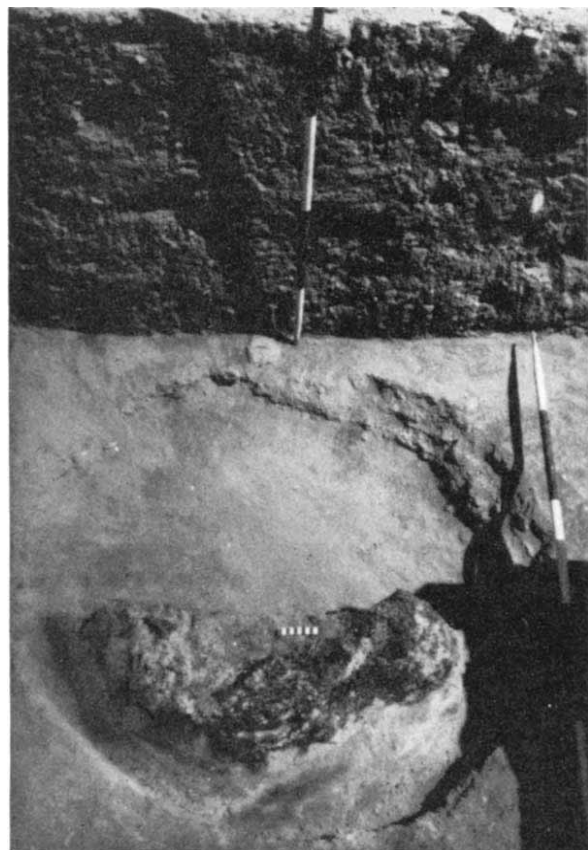


Fig. 2 Map of the area and the sites referred to in the text.

In the Jebel et Tomat excavations no evidence of dwelling or other structures was found, so these are most likely to have been built of temporary materials—grass or mats and poles—and to have been either easily transported (if the population was nomadic) or rebuilt every year, in either case without leaving any permanent record. Considering all the evidence available on the palaeoecology<sup>5</sup> and economy of Jebel et Tomat and other sites in central Sudan, it seems most probable that the Jebel et Tomat population of the early first millennium was transhumant, living along the Nile from mid-January to June

Fig. 3 Lower part of the storage pit dug into the dark clay loam, showing one half of the concentration of carbonised plant remains. Photo: J. D. C.



when they moved east with their herds to Jebel et Tomat to graze the Manaql ridge and the surrounding grasslands. Sorghum and, no doubt, other crops such as dukhn (*Pennisetum americanum*) and lubia (*Vigna* spp.) were planted in the summer growing season and reaped in November–December after which the people moved west to the Nile again. Such a pattern is not unlike that followed today.

The evidence from Jebel et Tomat is not alone in indicating the importance of sorghum in economies of Central Sudan in the early part of the present era. Sorghum, provisionally identified as the bicolor race (Eklass Abd El Bari, personal communication) has been found at Handal, a somewhat later site in Nubia. Also from Nubia comes a bouquet of sorghum

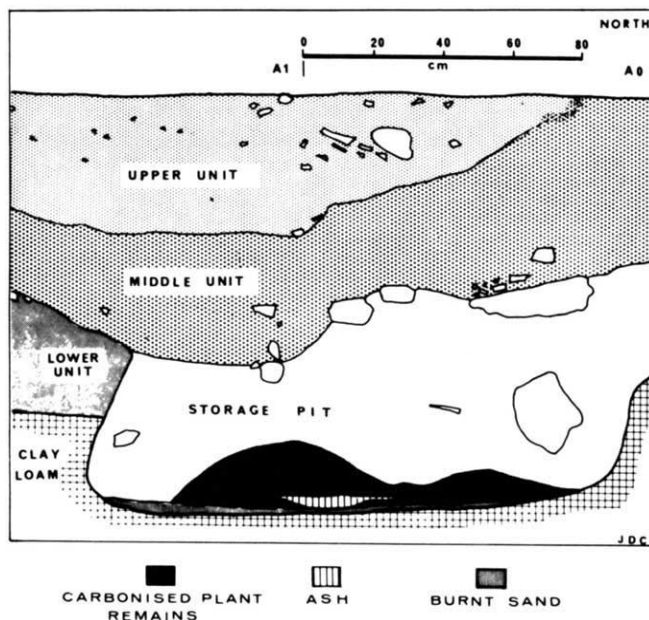


Fig. 4 North-South section across centre of storage pit containing carbonised Sorghum, Jebel et Tomat; excavation T.T.I.

inflorescences of race bicolor (identification verified by J. R. Harlan and J. M. J. de Wet, personal communication) found in a (?) ritual pit dug into a late Meroitic pavement at Qasr Ibrim<sup>6</sup>.

In the engraving of the Meroitic King Sherkarar (12–17 AD) at Jebel Qeili in the southern Butana, about 60 km north of Khartoum, heads of sorghum appear to be represented (ref. 7 pages 51 and 159) and millet, presumably sorghum, is referred to in particular by the geographer Strabo following his visit to Egypt in 25–24 BC. He stated: "The Ethiopians live on millet and barley from which they also make a drink..." He goes on to say "But some have grass as food, as also tender twigs, lotus and reed roots; and they use meats, blood, milk and cheese"<sup>8</sup>. This suggests that cereal cultivation had not yet become universal on the Nubian Nile and was probably subsidiary in importance to stock herding, as it is today.

Evidence cited above suggests that sorghum was a major crop of the Meroitic Kingdom, but how much earlier it was domesticated remains to be shown. Both Jebel et Tomat and Jebel Moya shared a number of cultural traits in common with what has been called the Butana Industry from Khashm el Girba in the Atbara drainage some 340 km to the east north-east where there are similar substantial midden accumulations at six sites. Site N125 is not yet excavated, but a charcoal sample (Tx445) from *in situ* in a test pit gave a result of  $4,410 \pm 90$  b.p. (2460 BC), in general agreement with the earlier dates at Jebel et Tomat and Jebel Moya<sup>9</sup>. All these sites belong to a single long lived cultural tradition that had its beginnings in the

early third millennium BC in the Khartoum Neolithic and that, in its later stage, shows a number of traits in common with Meroitic culture in the centuries immediately before and following the beginning of the present era. It is not inappropriate to term this the "Jebel Moya tradition" and, when a search for plant remains from the third millennium settlements is carried out, it would not be unexpected if an early form of domestic sorghum were found to be present.

We thank Rainer Berger for the radiocarbon dates; Gerald Wickens for charcoal identifications; Donald Adamson for identification of sponge spicules in the Jebel et Tomat pottery; Eklass Abd El Bari for information on her provisional identification of the Handal sorghum; and Peter Shinnie for information on the Nubian sites yielding sorghum. We also thank Andrew Smith, Daniel Stiles and Kenneth Williamson who took part in our excavations and were responsible for analysis of cultural and faunal material; Martin Williams and Donald Adamson for their help and close collaboration in the field; the Sudan Department of Antiquities and members of the Departments of Botany, Geology and Archaeology at the University of Khartoum; and Betty Clark for secretarial assistance. The archaeological investigation was made possible by grants from the British Academy and the Ford Foundation.

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- <sup>1</sup> Williams, M. A. J., and Adamson, D. A., *Geogr. J.*, **139**, 498–508 (1973).
- <sup>2</sup> Mukherjee, R., Rao, C., and Trevor, J. C., *The Ancient Inhabitants of Jebel Moya, Sudan* (Cambridge Univ. Press, 1955).
- <sup>3</sup> Addison, F., *Wellcome excavations in the Sudan: I, Jebel Moya, 1910–1914* (Oxford Univ. Press 1949).
- <sup>4</sup> Harlan, J. R., and de Wet, J. M. J., *Crop Sci.*, **12**, 172–176 (1972).
- <sup>5</sup> Williams, M. A. J., and Adamson, D. A., *Nature*, **284**, 584–586 (1974).
- <sup>6</sup> Plumley, J. M., *J. Egypt. Archaeol.*, **56**, 12–18 (1970).
- <sup>7</sup> Shinnie, P. L., *Meroe: A civilisation of the Sudan* (Thames and Hudson, London, 1967).
- <sup>8</sup> *The Geography of Strabo*, (edit. by Jones, H. L.), 143 (London, 1932).
- <sup>9</sup> Shiner, J. L., *The Prehistory and Geology of northern Sudan*, 335–394 (National Science Foundation, 1971).

## First direct evidence of life under Antarctic shelf ice

A TOPIC of current interest to Antarctic marine biologists is the possibility of a biome at considerable distances from the open sea under the vast, permanent ice shelves that fringe areas of Antarctica. Such biological information is one of the aims of the present Ross Ice Shelf project<sup>1</sup>, an attempt to drill through ice 500 m thick at 82°30'S, 166°00'W, a site 450 km from the ice front in the Ross Sea. We have already obtained, under unusual circumstances, direct evidence of a biome under shelf ice at least 100 km from the open sea.

During the 1973–74 austral summer we carried out a limnological survey between latitudes 70°48'S and 71°20'S on the west coast of Alexander Island (Fig. 1). King George VI Sound, which separates the island from the continent (Palmer Land), is covered by shelf ice 100–500 m thick<sup>2</sup>. The shelf ice receives glacier ice from both Palmer Land and Alexander Island. The resultant force is towards the northwest and rows of pressure ridges, 10–15 m high, lie along the west coast of Alexander Island in this latitude.

We caught four specimens of a common Antarctic marine benthic fish, *Trematomus bernacchii*, in a large (5 × 4 km) proglacial lake, lying in Ablation Valley (70°49'S, 68°25'W), (Fig. 2). The lake is over 117 m deep and has a permanent ice cover, 4.0–4.5 m thick in winter, 2.5–3.0 m thick in summer. The annual mean air temperature is approximately –9 °C. Ice

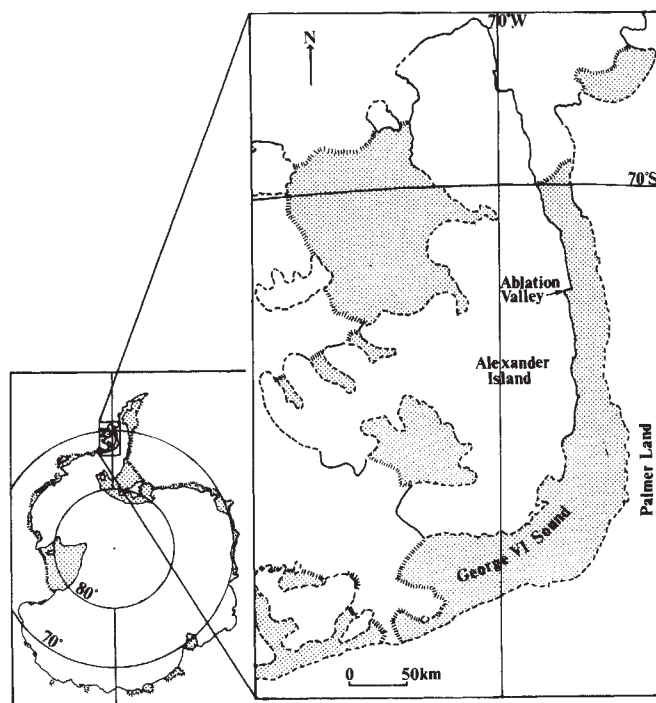


Fig. 1 Outline map of Alexander Island and its position in Antarctica. Ice coastline definitive/approximate is shown by continuous/dotted line. Ice shelf, stippled area. Front of ice shelf, dashed area.

from the Sound pushes into the lake along the 70 m depth contour for 3.4 km. Salinity measurements revealed that the top 55 m of water were fresh (0.1–1.0‰) but below 66.5 m the salinity was 32‰. The halocline was very steep and between 66.00 and 66.25 m the salinity rose from 18 to 31.5‰. The lake surface moved up and down with an irregular diurnal tidal rhythm. Variation in salinity profile with tidal movement indicated that the saline layer was in direct contact with the seawater of the Sound. The seawater extended for 4.0 km into the lake. Approximately 12% of available light entered the lake during the summer and we estimate that less than 0.05% reached the seawater layer. Heat gain by the water is restricted by the high latent thermal capacity of the lake and Sound ice, and the seawater. Temperatures remained almost constant throughout the period of investigation, the profile ranging from +0.10 °C to –1.60 °C (seawater). The oxygen content of the water was between 100 and 90% saturation in the freshwater layers, falling to 53% saturation in the seawater layer.

The fish were caught in a trap lying in 70 m of water. Seal meat was used as bait. The statistics of the fish are recorded in Table 1. The fish were in good condition although the weight-length ratios were lower than those recorded by Hureau<sup>3</sup> and Wohlschlag<sup>4</sup> for specimens captured in the equally cold waters off Terre Adelie and in McMurdo Sound. Only one of the Ablation Valley specimens had mature gonads, a female of 12.60 cm standard length. Hureau<sup>3</sup> found that females of the Terre Adelie population became mature when 17.50 cm standard length on average (recorded range 16.20–20.00 cm), males when 14.50 cm standard length (range not given, smallest 13.00 cm). The contrast is sufficient to suggest that the individuals caught in Ablation Valley are of a smaller growth form.

The stomachs of the fish contained the remains of nektonic, planktonic and benthic organisms (Table 2). Muscle tissue attached to crustacean skeletal material indicated that the fish had fed recently. The freshwater layer of the lake has been colonised by a calanoid copepod, *Pseudoboeckella* sp. (average concentration recorded 0.5 individuals l<sup>-1</sup>, including naupliar stages) but the remains removed from the fish were of different





Fig. 2 Aerial photograph of Ablation Valley. The lake occupies the whole of the wedge-shaped valley floor. King George VI Sound lies in the foreground. (Photography flown by the US Navy for the US Geological Survey).

and larger species. Phytoplankton production measurements by standard radiocarbon technique on 2 d at the height of the summer indicated that the amount of carbon fixed per day was about  $60 \text{ mg m}^{-2}$  for a zone 20 m deep. The only benthic vegetation seen during a SCUBA search covering about  $10,000 \text{ m}^2$  in the freshwater zone was a thin film of algae on the occasional rock jutting out of a silt floor. We conclude that the productivity of the freshwater layer is probably extremely low and insufficient to support a fish population. Furthermore the fish were not adapted to freshwater and showed signs of acute distress when brought to the surface.

A cyclopoid copepod, as yet unidentified, was collected below 40 m depth from water of salinity range 0.6–32‰. The maximum concentration recorded was 0.06 individuals  $\text{l}^{-1}$ , although only 67 m of tubing were available for the sampling pump and this only just reached the marine layer. Although the cyclopoids were able to tolerate fresh/brackish waters they were presumably more numerous at greater depths.

*T. bernacchii* is a slow-growing fish of very sedentary habit. Our evidence suggests that there is a marine biome under the

Table 1 Morphometric parameters of *T. bernacchii* from Ablation Valley

|                     | Specimen |        |        |        |
|---------------------|----------|--------|--------|--------|
| Sex                 | 1<br>♀   | 2<br>♀ | 3<br>♀ | 4<br>♂ |
| Size (cm)           |          |        |        |        |
| Total length        | 14.40    | 15.60  | 17.10  | 16.20  |
| Standard length     | 12.60    | 13.60  | 14.80  | 14.00  |
| Head length         | 3.90     | 4.23   | 4.87   | 4.26   |
| Head width          | 2.80     | 3.01   | 3.32   | *      |
| Head depth          | 2.78     | 2.58   | 2.97   | *      |
| Snout length        | 1.02     | 1.22   | 1.22   | 1.20   |
| Cheek height        | 0.36     | 0.34   | 0.33   | 0.34   |
| Interorbital width  | 0.42     | 0.38   | 0.51   | 0.55   |
| Orbit length        | 1.23     | 1.38   | 1.50   | 1.42   |
| Eye diameter        | 1.03     | 1.22   | 1.08   | 1.18   |
| Body depth          | 2.82     | 3.01   | 3.20   | 3.28   |
| Pectoral fin length | 3.05     | 2.93   | 2.89   | 3.13   |
| Pelvic fin length   | 2.45     | 2.88   | 3.46   | 3.10   |
| Weight (g)          |          |        |        |        |
| Total               | 36.85    | 43.30  | 60.90  | 47.05  |
| Gonad               | 0.9412   | 0.0633 | 0.0610 | 0.0634 |
| Fin-ray formula     |          |        |        |        |
| Dorsal              | V, 36    | V, 36  | V, 37  | V, 37  |
| Anal                | 33       | 33     | 33     | 32     |
| Pectoral            | 24       | 24     | 24     | 24     |

\* Specimen died with opercula raised.

Table 2 Gut contents

| Stomach                 | Intestine                                   |
|-------------------------|---------------------------------------------|
| Copepoda, calanoid spp. | Copepoda, Calanoid spp.                     |
| Gnathiidae, whole larva | Copepoda, harpacticoid sp.                  |
| Fish eye lenses         | Hydroid nematocysts                         |
| Fish scales, ctenoid    | Tanaid sp.                                  |
| Polychaetae spines (?)  | Compound eyes, decapod, mysid or euphausiid |
| Isopoda spp.            | Porifera spicules                           |

permanent ice of King George VI Sound, and in Ablation Valley which lies 100 km (northwards) and 334 km (southwards) from the open sea. There are two areas along the eastern shore of the Sound where the ice is fissured and seawater lies within a metre of the ice surface (Carse and Horse Points). Working through these 'holes' could provide further information on this remarkable biome.

We thank M. Macrae for the loan of his home-made fish-trap, A. Wheeler (British Museum) for identifying the fish, members of the Marine Section, British Antarctic Survey, for identifying some of the stomach contents, and Dr I. Everson for helpful comments.

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- 1 Zumbeke, J. H., *Antarct. J. U.S.*, 6, 258–63 (1971).
- 2 Smith, B. M. E., *Sci. Rep. Br. Antarct. Surv.*, 72, 1–11 (1972).
- 3 Hureau, J. C., *Bull. Inst. océanogr. Monaco*, 68, 1–244 (1970).
- 4 Wohlschlag, D. E., *Copeia*, 1, 11–18 (1961).

## Lack of response to background colour in *Pieris brassicae* pupae reared on carotenoid-free diet

THE Large White butterfly (*Pieris brassicae* L.) is a toxic species<sup>1</sup>, aposematic at all stages of its life-cycle. Probably for this reason, the pupa responds to the colour of its background in a somewhat different manner from that of many other Pierid species, displaying heterochromy (background contrasting) rather than homochromy (background matching). In this respect, there is some genetical variation between different strains (M. R., unpublished).

Gardiner<sup>2</sup> noted that in the Cambridge strain of this butterfly the diapausing pupa is invariably green, whereas most overwintering cryptic British butterfly pupae are brown, and those of the summer broods green (ref. 3 and Allister Smith, unpublished). Non-diapausing pupae of the Cambridge strain are more labile and respond to background and other environmental cues, by colour changes, varying from silvery white, grey and green to brown, with more or less extensive blotching and freckling with dark brown or black. Non-diapausing pupae of *Papilio polyxenes asterias* Stoll are also more labile<sup>4</sup> than diapausing pupae, which are consistently brown in this species.

When caterpillars of *P. brassicae* (Cambridge strain) are reared on standard artificial diet lacking cabbage<sup>5</sup> (Table 1), virtually devoid of carotenoids (but not vitamin A), both the resulting diapausing or non-diapausing pupae are invariably turquoise-blue in colour, freckled with small black spots (Class 1 of the Oltmer melanisation scale)<sup>6,7</sup>. The cuticular keel and spiracles are white (Table 1). In such specimens (several thousands have been tested) there is no visible response to background, whether the caterpillars or prepupae are exposed to long or short hours of daylight, dim or bright illumination, or reared on smooth-surfaced artificial diet, or on rough-surfaced artificial diet in containers covered with: coarse dark

**Table 1** Carotenoids in pupae of *P. brassicae* and their diets (selected examples\*)

| Food of larvae<br>(not diapausing)<br>at 70 °F                                                                                                       | Number and colour of<br>pupae and cuticle                                                                                                                        | Total<br>carotenoids<br>(µg per g dry weight) | Lutein<br>(µg per g dry weight)                   | Carotenoids<br>per insect<br>(µg) |
|------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|---------------------------------------------------|-----------------------------------|
| (1) Cabbage leaves<br>Greyhound cultivar<br>(2,750 µg per g carotenoids);<br>pupating on wood                                                        | 12 ♀ (whole)<br>Silver-grey; melanin Class 5 or blacker;<br>yellow cuticular keel                                                                                | 535.5                                         | 221.3 (41.6%)                                     | 17.75                             |
|                                                                                                                                                      | 13 specimens; cuticle and epidermis only;<br>same coloration                                                                                                     | 22.6                                          | 17.8 (78.8%)                                      | 0.24                              |
| (2) Artificial diet containing<br>xanthophyll extract;<br>(207 g per 100 g)<br>pupating on wood                                                      | 10 cuticles + epidermis<br>8 specimens, green; 2 specimens silver-grey;<br>melanin Class 5 or blacker;<br>yellow keel (indistinguishable from<br>control, No. 1) | 16.8                                          | 16.8 (100%)                                       | 0.15                              |
| (3) Artificial diet containing<br>wheat germ; pupating on wood                                                                                       | 9 specimens; Turquoise blue;<br>melanin Class 1; white keel                                                                                                      | 1.2                                           | ND                                                | 0.038                             |
| (4) Cabbage leaves<br>'January King' cultivar;<br>pupating on white netting at<br>sides of cage                                                      | 6 specimens (whole) Green;<br>melanin Class 1; yellow keel;<br>indistinguishable from No. 5                                                                      | 247.6                                         | 139.2 (56.2%)                                     | 10.7                              |
| (5) Artificial diet containing<br>wheat germ and xanthophyll<br>extract from cabbage and<br>β carotene pupating on white<br>netting on sides of cage | 6 specimens (whole); green;<br>melanin Class 1; yellow keel;<br>indistinguishable from control, No. 4                                                            | 286.8                                         | 162.4 (56.6%)                                     | 8.6                               |
| (6) Artificial diet containing<br>wheat germ; pupating on white<br>netting on sides of cage                                                          | 6 specimens (whole); turquoise-blue;<br>melanin Class 1; white keel                                                                                              | 13.7                                          | 13.7 (100%)                                       | 0.50                              |
| (7) Artificial diet containing<br>xanthophyll extract; reared and<br>pupating on glass                                                               | 20 cuticles + epidermis;<br>green; melanin Class 1; yellow keel                                                                                                  | 8.8                                           | 7.3 (83%)†<br>1.5 (5,6-mono-<br>epoxy-α-carotene) | 0.14                              |

\* Extraction methods were as described previously<sup>19</sup>. Other experiments with different cultivars and diets will be published elsewhere. The xanthophyll extract (NBC, Cleveland, Ohio) contained twelve or more different xanthophylls. Lutein made up only 1.4% of total carotenoids (5.4 mg per g dry weight), whereas 5, 6-mono-epoxy-α-carotene (69.7%) was the most abundant single xanthophyll in this extract. Wheat-germ, the overspill of manufactured Bemax, contained traces of four carotenoids—β carotene, lutein, zeaxanthin and flavoxanthin<sup>18</sup>. Hoffman LaRoche supplied β carotene, and Valadon and Mummery extracted the cabbage lutein.

† Contained 1.5 µg g<sup>-1</sup> 5,6-mono-epoxy-α-carotene (17%) as well as 83% lutein.

ND, not determined.

brown hessian, in deep shade; smooth, light-coloured natural wood, in bright summer sunlight; rough white bath towelling in winter sunlight; or in glass jam jars in various conditions.

If lutein is added to the artificial diet (0.5 ml lutein extracted from African marigold added to 100 g standard diet, (see Table 1)), the resulting pupae are bright green instead of turquoise-blue, with a yellow cuticular keel and spiracles, and Class I black freckling. Lutein can then be shown to be present in the epidermis and cuticle (Table 1). Of 40 such pupae, several responded to the background and developed melanisation to varying degrees (Classes 1–5). Moreover, two were indistinguishable, both in colour and pattern, from the silvery grey and heavily blackened cabbage-fed controls (derived from the same egg batches) pupating alongside them on the wooden walls of the breeding cage and its glass front.

Relatively small (but not less than 1 µg) concentrations of carotenoids per individual seem to be required to mediate background response, since those pupae reared on white cabbage (131.9 µg g<sup>-1</sup> carotenoids) contain only 1.58 µg of carotenoids per pupa, and are indistinguishable from green cabbage-reared specimens which each contain 12–19 µg per individual. Nevertheless, the 207 µg Xanthophyll per 100 g diet (Table 1) must be close to the lower limit which enables biliverdin and the carotenoids themselves to be partially excluded from the cuticle and epidermis<sup>8,9</sup>, since only 6% of these lutein-reared pupae exactly matched the typical silvery grey and black coloration of the controls.

It has been generally accepted<sup>8–11</sup> that the colour and

pattern of *Pieris* and various other Lepidoptera pupae are controlled or modified by endocrine factors released from the anterior end of the body in response to a formidable and confusing array of sensory cues and biological mechanisms. It has been suggested that the melanisation of the cuticle and the distribution of ommochromes in the tissues of *P. brassicae* are controlled by different hormones from those which control the presence or distribution of bile pigments and the carotenoids themselves. Kayser and Angersbach<sup>8</sup>, however, believe that the degree of melanisation and bile pigment content are regulated by the same hormonal factor. Our experiments indicate that at least in the case of the Cambridge strain of *P. brassicae*, carotenoids—possibly only lutein—function as a mediator, without which light cues associated with the insects' ability to respond to background do not operate. In the absence of dietary carotenoids the blue bile pigments<sup>12,13</sup> synthesised and released during the fifth larval instar and the 'sensitive' prepupal stage<sup>8,14</sup> remain in the haemolymph and epidermis and are neither re-routed nor degraded as in those pupae which turn silvery grey and black.

It is possible that carotenoids play some role in determining the colour modifications known to be associated with photoperiod, and characteristic of spring and summer broods of many Pierid butterflies<sup>15–17</sup>. It is also worth considering the influence of these substances in examples of circadian rhythms which, it has been suggested, could involve a photosensitive molecule participating in the clock mechanism<sup>18</sup>. In any case careful note should be taken of the carotenoid content of pupae and larvae



of Lepidoptera, whether reared on natural or artificial diet, which are used in experiments involving light cues.

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- <sup>1</sup> Marsh, N., and Rothschild, M., *J. Zool. Lond.*, **174**, 89–122 (1974).
- <sup>2</sup> Gardiner, B. O. C., *Wilhelm Roux Arch. EntwMech. Org.*, **176**, 13–22 (1974).
- <sup>3</sup> Cott, H. B., *Adaptive Coloration in Animals* (Methuen, London, 1940).
- <sup>4</sup> West, D. A., Snellings, W. M., and Herbek, T. A., *Jl N.Y. ent. Soc.*, **80**, 205–211 (1972).
- <sup>5</sup> David, W. A. L., and Gardiner, B. O. C., *Bull. ent. Res.*, **53**, 91–109 (1962).
- <sup>6</sup> Oltmer, A., *Wilhelm Roux Arch. EntwMech. Org.*, **160**, 401–427 (1968).
- <sup>7</sup> Bückmann, D., *Wilhelm Roux Arch. EntwMech. Org.*, **166**, 236–253 (1971).
- <sup>8</sup> Kayser, H., and Angersbach, D., *J. Insect Physiol.*, **20**, 2277–2285 (1974).
- <sup>9</sup> Kayser, H., *J. Insect. Physiol.*, **20**, 89–103 (1974).
- <sup>10</sup> Joly, P., *Endocrinologie des Insectes* (Masson, Paris, 1968).
- <sup>11</sup> Fuzeau-Braesch, S., *A. Rev. Ent.*, **17**, 403–424 (1972).
- <sup>12</sup> Rüdiger, W., Klose, W., Vuillaume, M., and Barbier, M., *Experientia*, **24**, 1000 (1968).
- <sup>13</sup> Cherbas, P. T., thesis, Harvard Univ. (1973).
- <sup>14</sup> Van der Geest, L. P. S., *J. Insect Physiol.*, **14**, 537–542 (1968).
- <sup>15</sup> Watt, W. B., *Evolution*, Lancaster, Pa., **22**, 437–458 (1968).
- <sup>16</sup> Watt, W. B., *Proc. natn. Acad. Sci. U.S.A.*, **63**, 767–774 (1969).
- <sup>17</sup> Hoffman, R. J., *Evolution*, Lancaster, Pa., **27**, 387 (1973).
- <sup>18</sup> Truman, J. W., *Proc. int. Symp. circadian Rhythmicity*, Wageningen, **111–135** (1971).
- <sup>19</sup> Feltwell, J., and Rothschild, M., *J. Zool., Lond.*, **174**, 441–465 (1974).

## Mass production of marine algae in outdoor cultures

TWENTY-FIVE years ago the large scale culturing of unicellular algae was viewed with great enthusiasm as an alternative method for producing protein<sup>1</sup>. This hope diminished by the late 1960s when it seemed that the process was uneconomical because of a combination of technical problems, most notably the recovery of the algal product and its subsequent conversion to a human food and/or food supplement.

Quite recently though, there has been renewed interest in producing single-celled protein by mass culturing unicellular algae. This interest has been stimulated in part by the demonstration that algal systems can be used for both pollution control and protein production by directly recycling the nutrients in wastewater into the biomass of freshwater algae<sup>2</sup>. Recently, it has been shown that a marine counterpart, in which algae are grown on mixtures of wastewater and seawater, can serve the same roles with distinct advantages over freshwater algal cultures<sup>3</sup>.

The algae are readily removed by utilising the microscopic organisms as the first link in marine food chains leading to multicomponent and integrated aquaculture systems<sup>4</sup>.

As part of our efforts in developing the above process, we have been experimenting outdoors with continuous-flow algal pond cultures, trying to optimise biomass yields. We report here the results of long term experiments conducted during the past two years at Woods Hole, Massachusetts (May–October 1973) and Ft Pierce, Florida (May–October 1974).

The experiments were performed on the Woods Hole Oceanographic Institution dock and adjacent to the Harbor Branch Foundation Laboratory at Ft Pierce. The culture

**Table 1** Effect of dilution rate on mean algal yield in two locations

| Dilution rate | Mean algal yield (g dry weight m <sup>-2</sup> d <sup>-1</sup> ) |                             |
|---------------|------------------------------------------------------------------|-----------------------------|
|               | Woods Hole<br>(lat. 41°52'N)                                     | Ft Pierce<br>(lat. 27°28'N) |
| 0.25          | 7.6 (2)                                                          |                             |
| 0.50          | 12.4 (8)                                                         | 18.0 (6)                    |
| 0.75          | 13.3 (2)                                                         | 15.3 (1)                    |
| 1.00          | 12.4 (1)                                                         | 23.6 (1)                    |
| 1.50          |                                                                  | 0* (1)                      |

Values in parentheses represent number of replicate experiments. Each experiment consisted of determining the steady-state algal yield at the stated dilution rate. The mean algal yields, where applicable were determined by averaging the data from the replicates. Variations from the means were less than  $\pm 20\%$ .

\* Steady-state algal growth could not be maintained and the culture washed out.

units were two replicate ponds each of 2,000-l capacity (2.3 m diameter and 50 cm deep) and lined with glass fibre. Secondly treated domestic wastewater was obtained daily from local treatment facilities and stored in polyethylene tanks. Seawater was pumped continuously through 1- $\mu$ m filters and blended with the wastewater into the ponds at the desired flow rates and mixture. The ponds were well mixed continuously with rotating arms and by recirculation through pumping so that they resembled completely mixed reactors.

Algal yield in a continuous culture is defined as the product of biomass concentration and flow rate on a per unit surface area basis. Thus by varying the dilution rate (fraction of volume turnover per day) in these studies from 0.25 to 1.50, we were able to determine the maximum attainable areal yields. For each dilution rate we establish relative steady-state conditions by maintaining a constant flow rate for 7–10 d.

A true steady state was impossible to establish because algal biomass concentrations were affected by daily variations in sunlight intensity and pond temperatures. Once relative steady-state conditions were established, however, there was no more than a  $\pm 10\%$  daily change in biomass concentration. In both studies we added a mixture of 50% wastewater and 50% seawater to the ponds and continuously monitored inorganic nitrogen and phosphorus concentrations to ensure that nutrients never limited algal growth. We estimated algal biomass by measuring particulate carbon concentrations on a Perkin-Elmer 240 Elemental Analyzer; we then converted these values to ash-free dry weights by assuming that carbon comprises 45% of the total organic matter in algae, following experimental determination of this relationship.

Our results indicate that algal yields were relatively constant between dilution rates of 0.50 and 1.00 (average of 12.7 g dry weight m<sup>-2</sup> d<sup>-1</sup> in the Woods Hole study and 19 g dry weight m<sup>-2</sup> d<sup>-1</sup> in the Ft Pierce study), but were significantly reduced (7.6 g dry weight m<sup>-2</sup> d<sup>-1</sup>) at a dilution rate of 0.25 and were zero (cell washout) when the dilution rate was 1.50 (Table 1). The experiments at each location had to be spread over the May–October study periods; therefore, effects of seasonal changes in sunlight intensity and temperature were not eliminated as experimental variables. Some of the variations in the results, both in replicate experiments and at different dilution rates (Table 1), could be accounted for by changes in these environmental factors.

In both studies we did not inoculate the ponds with a particular algal species. Rather, when the ponds were first filled with wastewater–seawater mixtures, virtually a monoculture of a marine diatom species was quickly established from the indigenous algae present in the seawater. In the Woods Hole experiments *Phaeodactylum tricornutum* dominated during May and June, followed by *Amphiprora* sp. which prevailed until late August when *Amphora* sp.

became dominant. In late September through October *Amphiprora* sp. returned as the dominant alga. During the entire Ft Pierce experiment *Nitzschia closterium* was the main diatom.

We feel that our results are particularly noteworthy because they represent the first reported experiments on successful mass production of marine algae in fully continuous outdoor cultures that were maintained with little difficulty for the entire study periods indicated. In contrast, virtually all the data available on yields in outdoor cultures of marine and freshwater algae have been attained by intermittent collection in semicontinuous or batch cultures<sup>5-8</sup>. We have also shown the effects of geographical location on algal yields—the average yields at Ft Pierce were more than 50% greater than the average achieved at Woods Hole.

Our results compare favourably with yields of 10–20 g dry weight  $m^{-2} d^{-1}$  that have been reported for freshwater algae in several European studies<sup>5,6</sup> and are considerably better than the 5–12 g dry weight  $m^{-2} d^{-1}$  attained so far in marine mass cultures<sup>7,8</sup>. They are consistent with Ryther's original estimate<sup>9</sup> that, based on sunlight availability only, a maximum yield of about 27 g dry weight  $m^{-2} d^{-1}$  can be sustained in a water body. It seems then not to be possible to achieve yields much above the values we have attained. As has been shown<sup>9,10</sup>, these production values are comparable with those for the fastest growing agricultural crops such as sugar and rice.

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<sup>1</sup> Burlew, J. S., *Algal culture from laboratory to pilot plant*. Carnegie Institution of Washington Publication 600 (Carnegie Institution, Washington, DC, 1953).

<sup>2</sup> Oswald, W. J., *Chem. Engn. Prog. Symp. Ser.*, **65**, 87–92 (1969).

<sup>3</sup> Ryther, J. H., Dunstan, W. M., Tenore, K. R., and Huguenin, J. E., *Bioscience*, **22**, 144–152 (1972).

<sup>4</sup> Goldman, J. C., Tenore, K. R., Ryther, J. H., and Corwin, N., *Water Res.*, **8**, 45–54 (1974).

<sup>5</sup> Vendlova, J., *Annali Microbiol.*, **19**, 1–12 (1969).

<sup>6</sup> Jaleel, S. A., and Soeder, C. J., *Indian Food Packer*, **27**, 45–57 (1973).

<sup>7</sup> Ansell, A. D., Raymont, J. E. G., Lander, K. F., Crowley, E., and Shackley, P., *Limnol. Oceanogr.*, **8**, 184–206 (1963).

<sup>8</sup> Dunstan, W. M., and Tenore, K. R., *Aquaculture*, **1**, 181–192 (1972).

<sup>9</sup> Ryther, J. H., *Science*, **130**, 602–608 (1959).

<sup>10</sup> Westlake, D. F., *Biol. Rev.*, **38**, 385–425 (1963).

## Genetic control of diploid-like meiosis in hexaploid tall fescue

ALLOPOLYPLOIDY has played a vital role in the evolution of plant species useful to man. Hybridisation between related species followed by chromosome doubling has given rise to some of our most important grain, forage and fibre crops. The successful establishment of a sexually reproducing polyploid would, however, depend on the integration of the constituent genomes into a meiotically and, hence, reproductively stable form which could be achieved only by means of a precise diploidising mechanism. In the case of bread-wheat it has been conclusively demonstrated that diploid-like meiotic behaviour is genetically controlled<sup>1,2</sup> and there is a strong indication of a similar control in other crop species, for example, hexaploid oats<sup>3</sup> and tetraploid cottons<sup>4</sup>.

Tall fescue (*Festuca arundinacea* Schreb.) is an allohexaploid ( $2n = 6x = 42$ ) comprising diploid genomes of three related diploid species of the *Lolium-Festuca* complex. That the three genomes are homoeologous is borne out by the fact that there is extensive, in some cases complete, pairing in the diploid, auto-

allotriploid and trispecific hybrids between the putative progenitors<sup>5</sup> and that some monosomics isolated by the author are fully fertile and morphologically indistinguishable from the sister euploid plants. Thus, although it contains three closely related homoeologous genomes, tall fescue regularly forms 21 bivalents between homologous chromosomes (Fig. 1a) indicating the possibility of genetic control of bivalent pairing. The evidence which strongly points to the presence of such a control is discussed here.

In a cross between the Algerian (*Bn* 273) and Israeli (*Bn* 488) ecotypes of tall fescue, all the five euploid ( $2n = 42$ ) hybrids studied formed 21<sub>II</sub>. The four euploids from the reciprocal cross (*Bn* 488 × *Bn* 273) also regularly formed 21<sub>II</sub> (Fig. 1a). A sister plant which was a monosomic ( $2n = 41$ ) numbered 90–64–7 formed some trivalents, quadrivalents (Fig. 1b), pentavalents and hexavalents, in addition to bivalents (Table 1). Another three monosomic plants of different genotypic background showed normal bivalent pairing. As all the euploid progeny from the cross *Bn* 273 × *Bn* 488 regularly formed 21<sub>II</sub> and the other monosomic lines had regular chromosome pairing (Table 1), it is reasonable to conclude that the missing chromosome in the monosomic 90–64–7 is critical for normal bivalent pairing. The consistent formation of 21<sub>II</sub> in the parents of this cross and in all the euploid hybrids rules out the possibility of the occurrence of translocations in the monosomic 90–64–7. The occurrence of a hexavalent, or a VI + IV or III, or V + IV or III, or IV + III, or 3 IV in the same cell would further discount the translocation hypothesis. The monosome itself was also sometimes involved in multivalent formation. It can therefore be inferred that multivalents in the monosomic 90–64–7 resulted from homoeologous pairing which was presumably caused by the removal of 'genetic regulator' on the missing chromosome. On this premise it seems that the genetic system controlling bivalent pairing in the hexaploid tall fescue is not effective in the hemizygous state, or at least, is not as effective as in the diploid state.

Clearly, in the other three bivalent-forming monosomics (Table 1), genetic information for the regulation of pairing was not located on the missing chromosome. For want of a full series of monosomics and nullisomics in tall fescue, however, it is not at present possible to conclude that the gene(s) system regulating bivalent pairing is solely confined to this particular chromosome involved in the monosomic 90–64–7.

The above inference receives substantial support from the

Fig. 1 a–c, Chromosome pairing in euploid and monosomic *F. arundinacea* *Bn* 488 × *Bn* 273; a, euploid hybrid with 21<sub>II</sub>; b, monosomic 90–64–7 with 2<sub>IV</sub> (arrowed) + 16<sub>II</sub> + 1<sub>I</sub> (note stickiness); c, monosomic 90–64–7 with 20<sub>II</sub> + 1<sub>I</sub> showing secondary associations of bivalents into groups of three or two bivalents, lower centre group of five bivalents and the univalent (arrowed); d, *L. perenne* × *F. rubra* hybrid with 12<sub>II</sub> + 4<sub>I</sub>; note five ring bivalents.

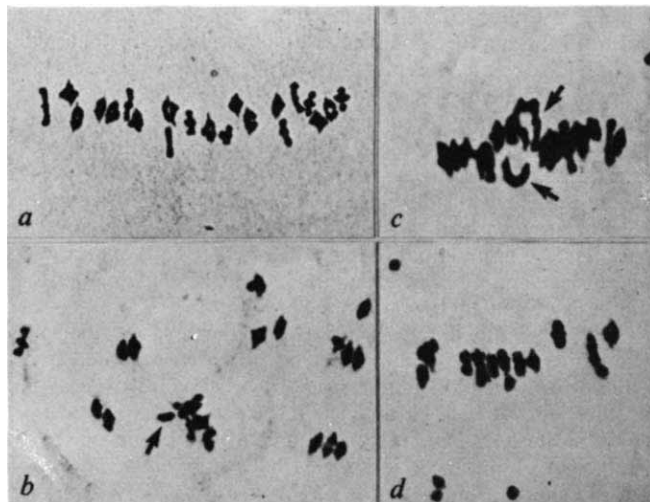




Table 1 Chromosome pairing in some euploid and monosomic plants of *Festuca arundinacea*

| Cross                           | 2n | VI   | V    | IV   | III  | II    | I    | Chiasmata per cell | No. of cells analysed |
|---------------------------------|----|------|------|------|------|-------|------|--------------------|-----------------------|
| <i>Bn 273</i> × <i>Bn 488</i>   |    |      |      |      |      |       |      |                    |                       |
| Hybrid -1                       | 42 | —    | —    | —    | —    | 21.00 | —    | 32.83              | 30                    |
| -2                              | 42 | —    | —    | —    | —    | 21.00 | —    | 33.40              | 30                    |
| -3                              | 42 | —    | —    | —    | —    | 20.90 | 0.20 | 30.97              | 30                    |
| -4                              | 42 | —    | —    | —    | —    | 20.97 | 0.07 | 30.83              | 30                    |
| -5                              | 42 | —    | —    | —    | —    | 20.97 | 0.07 | 31.80              | 30                    |
| <i>Bn 488</i> × <i>Bn 273</i>   |    |      |      |      |      |       |      |                    |                       |
| Hybrid-2                        | 42 | —    | —    | —    | —    | 21.00 | —    | 31.00              | 20                    |
| -3                              | 42 | —    | —    | —    | —    | 20.90 | 0.20 | 29.93              | 40                    |
| -6                              | 42 | —    | —    | —    | —    | 20.97 | 0.07 | 30.90              | 30                    |
| Monosomic 90-64-7               | 41 | 0.04 | 0.03 | 0.27 | 0.20 | 18.80 | 1.31 | 32.93              | 96                    |
| -9                              | 42 | —    | —    | —    | —    | 20.96 | 0.08 | 36.01              | 70                    |
| Other monosomics in tall fescue |    |      |      |      |      |       |      |                    |                       |
| (i) 1-15-4                      | 41 | —    | —    | —    | —    | 19.93 | 1.13 | 31.33              | 30                    |
| (ii) 17-78-2                    | 41 | —    | —    | —    | —    | 19.84 | 1.31 | 33.80              | 45                    |
| (iii) 19-21-9                   | 41 | —    | —    | —    | —    | 19.75 | 1.50 | 33.13              | 8                     |

*Bn 273*, Tall fescue from Algeria.

*Bn 488*, Tall fescue from Israel.

study of meiosis in the tall fescue polyhaploid ( $2n = 21$ ) in which a mean of  $4.01_{II}$  and  $12.96_I$  were observed with  $7_{II}$  in nearly 18% of the cells analysed<sup>6</sup>. These bivalents and a trivalent must have resulted from homoeologous pairing and is in accordance with the theory that the genetic system controlling bivalent pairing is ineffective in the hemizygous state. On the contrary, in wheat<sup>1</sup> and oats<sup>3</sup>, the polyhaploids show very little chiasmate pairing because genetic control is effective in the hemizygous state. It also seems that the regulator in the hemizygous state in the monosomic 90-64-7 permits multivalent formation without markedly affecting chiasma frequency (Table 1).

It is interesting to note that there was a conspicuous tendency for secondary associations among bivalents in the monosomic 90-64-7. Chromosome spreading was more difficult because of stickiness (Fig. 1b), but even in well spread metaphase plates the secondary associations were clearly evident (Fig. 1c). Since secondary associations are now known to take place between bivalents of genetically and evolutionarily related chromosomes<sup>7</sup>, it is reasonable to infer that the grouped bivalents have related chromosomes with synaptic potentiality and that they tend to be closely associated, presumably as a result of the relaxation of genetic control in this monosomic.

Further support for the existence of a regulatory system in tall fescue, which is not effective in its haploid complement, comes from the study of several other hybrids and amphiploids between tall fescue and diploid *Lolium* species. Thus, in the 28-chromosome hybrids between *L. multiflorum* ( $2n = 14$ ) and *F. arundinacea* ( $2n = 42$ ), Lewis<sup>8</sup> observed a maximum of  $14_{II}$  in several cells, whereas the mean for three hybrids was  $9.52_{II}$  per cell. Some of these bivalents, and associated trivalents and quadrivalents must have resulted from extensive homoeologous pairing resulting from the breakdown of the regulatory mechanism in the haploid complement of tall fescue. In the amphiploids ( $2n = 56$ ), however, when its full complement was present, and hence the regulator in the double dose, normal chromosome pairing was largely restored. Up to  $28_{II}$  (mean of three amphiploids =  $24.23_{II}$  per cell) were observed and the multivalent frequency was much lower compared to the 28-chromosome hybrids. Since the amount of differentiation between *Lolium* and *Festuca* chromosomes is very low and permits little preferential pairing<sup>5</sup>, the synaptic behaviour of the *multiflorum*-*arundinacea* amphiploids can be explained only on the basis of genetic regulation.

As far as the author is aware, a hemizygous ineffective regulator of diploid-like pairing has not been recorded in any other polyploids of Gramineae or any other family. It may well be, however, of wide occurrence in the grass family. That other polyploid fescues have a similar regulator is borne out by the study of two intergeneric hybrids between *Lolium perenne* L. ( $2n = 14$ ) and *Festuca rubra* L. ( $2n = 6x = 42$ ). Whereas the

parents formed  $7_{II}$  and  $21_{II}$  respectively, the hybrids (Fig. 1d) showed a mean of  $0.03_{IV} + 0.10_{III} + 11.57_{II} + 4.69_I$ . Twenty-eight per cent of the cells had 13 or 14 bivalents, and the frequency of ring-bivalents was high (mean chiasmata = 19.85 per cell). It seems that there was extensive homoeologous pairing as a result of the hemizygous state of the regulator in the haploid *rubra* complement in these hybrids. In the hexaploid *rubra*, however, no homoeologous pairing is observed because of the fully effective regulator in double dose.

Similar results were obtained in another intergeneric cross between *L. multiflorum* ( $2n = 14$ ) and *F. arundinacea* var. *letourneuxiana* ( $2n = 70$ ) and its amphiploid<sup>9</sup>. Whereas meiosis in the parents is characterised by bivalent formation, the hybrids ( $2n = 42$ ) formed a mean of  $13.72_{II}$ , and several trivalents, quadrivalents, pentavalents and hexavalents. The amphiploid ( $2n = 84$ ), however, showed almost regular meiosis forming predominantly bivalents (mean  $38.27_{II}$  per cell, some cells with  $42_{II}$ ). These results of Malik and Thomas<sup>9</sup> and those of Chandrasekharan and Thomas<sup>10</sup> and of several earlier workers like Crowder<sup>11</sup> and Lewis<sup>8</sup> can be more meaningfully explained on the basis of the existence of a 'genetic regulator' in tall fescue and other polyploid fescues, which exercises its regulatory control on homoeologous pairing when it is present in the double dose but is haplo-insufficient.

The hemizygous ineffectiveness of the regulator is clearly of evolutionary significance in that, in the hybrids, it could have permitted the gene flow from one species to another, but subsequent chromosome doubling resulted in stable amphiploids in nature. This would at least partly account for the widespread introgression of characters between taxa.

The possible existence of such a regulator could have important implications for the cytogenetic relationships of the entire *Lolium-Festuca* complex and the strategy of the plant breeder in making use of this group. To avoid loss of the 'genetic regulator' even on one chromosome, more emphasis will have to be laid on incorporating the full complement of hexaploid fescues, for example, tall fescue and red fescue, in the production of agronomically superior, stable *Lolium-Festuca* amphiploids. The precise location of the regulator in tall fescue and other polyploids in the Festuceae could open up new avenues in forage breeding of the type being witnessed in wheat<sup>12,13</sup>.

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<sup>1</sup> Riley, R., and Chapman, V., *Nature*, **182**, 713-715 (1958).

<sup>2</sup> Sears, E. R., and Okamoto, M., *Proc. tenth Int. Congr. Genet.*, **2**, 258-259 (1958).

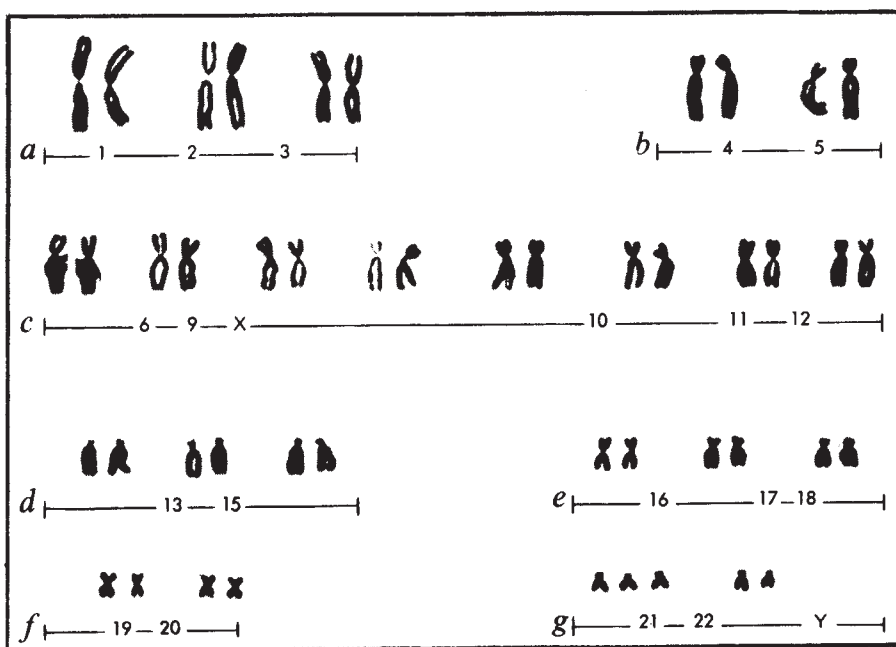
- <sup>3</sup> Rajhathy, T., and Thomas, H., *Nature new Biol.*, **239**, 217–219 (1972).
- <sup>4</sup> Kimbar, G., *Nature*, **191**, 98–100 (1961).
- <sup>5</sup> Jauhar, P. P., *Chromosomes Today*, **5** (in the press).
- <sup>6</sup> Malik, C. P., and Piprathi, R. C., *Zeits. Biol.*, **116**, 332–339 (1970).
- <sup>7</sup> Riley, R. and Law, C. N., *Adv. Genet.*, **13**, 57–114 (1965).
- <sup>8</sup> Lewis, E. J., *Proc. tenth Int. Grassl. Congr.*, 688–693 (1966).
- <sup>9</sup> Malik, C. P., and Thomas, P. T., *Z. Pflanzenzüchtg.*, **55**, 81–94 (1966).
- <sup>10</sup> Chandrasekharan, P., and Thomas, H., *Z. Pflanzenzüchtg.*, **65**, 345–354 (1971); **66**, 76–86 (1971).
- <sup>11</sup> Crowder, L. V., *J. Hered.*, **44**, 195–203 (1953); *Bot. Gaz.*, **117**, 220 (1956).
- <sup>12</sup> Riley, R., Chapman, V., and Johnson, R., *Genet. Res.*, **12**, 199–219 (1968).
- <sup>13</sup> Sears, E. R., *Stadler Symp.*, **4**, 23–38 (1972).

TERATOMAS are usually differentiated tumours, containing tissues derived from ectoderm, endoderm and mesoderm, and may arise in the gonads or elsewhere. Benign ovarian teratomas are parthenogenetic tumours derived from a single germ cell after the first division of meiosis, as studies of chromosome markers and enzyme variants have shown<sup>1,2</sup>. We have now found that extragonadal teratomas develop in a different manner. We have examined single gene products and chromosomes in extragonadal teratomas and report here our results. We include data on endodermal sinus tumours because of their similarity to teratomas<sup>3,4</sup>.

The results showed that in four cases normal host tissue was heterozygous at seven loci (Table 1). In all seven situations when the host was heterozygous the tumour was also heterozygous.

Chromosome studies showed that the teratoma of case 3, the patient with Down's syndrome, had trisomy 21 (47,XX,+21) (Fig. 1). If this tumour had been derived from a germinal cell after meiosis, two or four doses of chromosome 21 would have been expected. But there were three doses, as anticipated with normal mitosis. The 46,XY karyotype in the endodermal sinus tumour from a male (case 5) is likewise consistent with mitosis rather than meiosis at the time of inception of the tumour. A 46,XY chromosome constitution has also been observed<sup>6</sup> in a benign sacrococcygeal teratoma.

Thus all our results suggest that these tumours develop from a mitotic cell. The cell of origin could be either a somatic cell or a



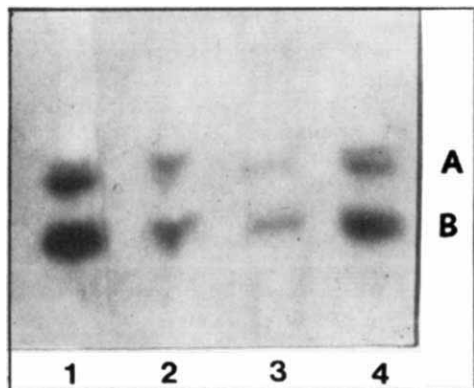
**Fig. 1** Karyotype analysis of cultured cells from a retroperitoneal teratoma in a person with Down's syndrome. The normal host cells and those of the teratoma are 47,XX,+21.

Tissues were grown in monolayer cultures and the electro-

G-6-PD data may shed light on the question of whether the tumours have a unicellular or multicellular origin. This enzyme is X-linked<sup>7</sup>, and in normal female cells one of the two alleles (*A* or *B*) undergoes random, irreversible inactivation<sup>8,9</sup> early in

**Table 1** Phenotype of extragonadal teratomas and endodermal sinus tumours[illegible]





**Fig. 2** Starch gel electrophoresis of G-6-PD isozymes performed on cell cultures. Normal host cells (1,2) and cells from a sacrococcygeal teratoma (3,4) are heterozygous with an AB phenotype.

embryonic development<sup>10</sup>. G-6-PD heterozygotes with an AB phenotype have two populations of somatic cells, one with the A and the other with the B phenotype. Some tumours such as leiomyomas of the uterus manifest only one G-6-PD phenotype (A or B), suggesting their origin from a single cell<sup>11</sup>. Tumours with a heterozygous G-6-PD phenotype<sup>12,13</sup> are thought to have originated from two or more cells.

The G-6-PD phenotype of the host in case 1 was AB, and so was her sacrococcygeal teratoma (Fig. 2). This teratoma could have originated from several cells, at least one of which was A and the other B. This presupposes that the tumour arose after X-inactivation. The times of X-inactivation and tumour inception in man are, however, too imprecise to know which came first: X-inactivation or the embryonal tumour. Another possibility therefore is that the tumour arose from a single cell before X-inactivation and that by chance in some cells the A allele was inactivated, while in others it was the B allele. A third possibility—that the teratoma arose from a cell with both G-6-PD alleles active—can be excluded, since no AB hybrid band was observed<sup>14,15</sup>.

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- <sup>1</sup> Linder, D., and Power, J., *Ann. Human. Genet.*, **34**, 21 (1970).
- <sup>2</sup> Linder, D., McCaw, B. K., and Hecht, F., *New Engl. J. Med.*, **292**, 63 (1975).
- <sup>3</sup> Teilum, G., *Acta path. microbiol. Scand.*, **64**, 407 (1965).
- <sup>4</sup> Mostofi, F. K., and Price, E. B., Jr., *Tumors of the male genital system* (Armed Forces Institute of Pathology, Washington, DC, 1973).
- <sup>5</sup> Terasaki, P., *Manual of tissue typing techniques* (edit. by Ray, J. G., Scott, R. C., Hare, D. B., Harris, C. E., and Kayhoe, D. E.), 50 (National Institutes of Health, Bethesda, Maryland, 1972).
- <sup>6</sup> Laurent, M., Rousseau, M.-F., and Nezelof, C., *Ann. d'Anatomie pathol.*, **13**, 413 (1968).
- <sup>7</sup> Childs, B., Zinkham, W., Browne, E. A., Kimbro, E. L., and Torbert, J.V., *Bull. Johns Hopkins Hosp.*, **102**, 21 (1958).
- <sup>8</sup> Beutler, E., Yeh, M., and Fairbanks, V. F., *Proc. natn. Acad. Sci. U.S.A.*, **48**, 9 (1962).
- <sup>9</sup> Davidson, R. G., Nitowsky, H. M., and Childs, B., *Proc. natn. Acad. Sci. U.S.A.*, **50**, 481 (1963).
- <sup>10</sup> Epstein, C. J., *Science*, **163**, 1078 (1969).
- <sup>11</sup> Linder, D., and Gartler, S. M., *Science*, **150**, 67 (1965).
- <sup>12</sup> Beutler, E., Collins, Z., and Irwin, L. E., *New Engl. J. Med.*, **276**, 389 (1967).
- <sup>13</sup> Gartler, S. M., Ziprkowsky, L., Krakowski, A., Ezra, R., Szeinberg, A., and Adam, A., *Am. J. hum. Genet.*, **18**, 282 (1966).
- <sup>14</sup> Yoshida, A., Steinmann, L., and Harbert, P., *Nature*, **216**, 275 (1967).
- <sup>15</sup> Gartler, S. M., Liskay, R. M., Campbell, B. K., Sparkes, R., and Gant, N., *Cell Differentiation*, **1**, 215 (1972).

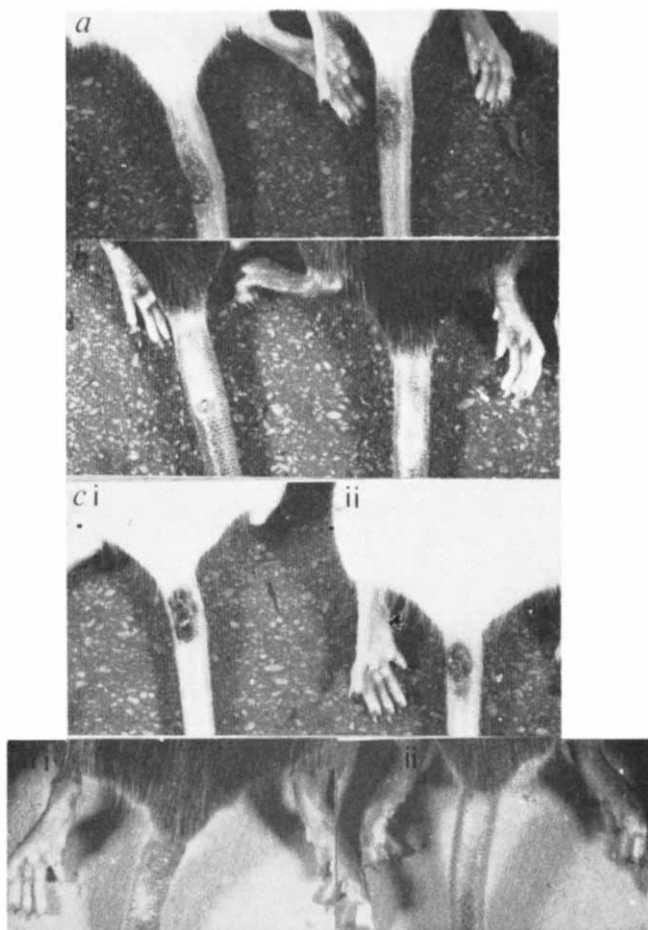
## Reaction of mouse strains to skin test for ectromelia using an allied virus as inoculum

ECTROMELIA, or 'mouse pox', is a highly infectious virus disease of mice which can be present as a subclinical enzootic or a clinically apparent epizootic condition. As a result of importation of infected mice, an outbreak of this disease occurred in research establishments in the London area during late 1973 and the early part of 1974. These mice showed immunological evidence of infection without overt signs of disease, and those in contact were affected by the acute form of the infection. Detection of the infection is usually effected by one of three methods: inoculation of suspect material on to the choriollantoic membrane of 10-d-old chick embryos, in which typical pocks lesions are produced 3 d later if virus is present; serologically, using haemagglutination test<sup>1</sup>; using the tail scarification test with vaccinia virus, a closely related pox virus, as inoculum.

During the outbreak, screening of large numbers of mice by the Laboratory Animals Centre (LAC) was carried out using the tail scarification method as the principal test. Marked variations in response to inoculation were observed between the different inbred strains screened. Briody<sup>1</sup> gave  $1.0 \times 10^4$  pock-forming units (PFU) of IDH-E strain of vaccinia as a vaccination dose and noted that reaction to the inoculation varies with the strain at this level of infection, but that at  $1.0 \times 10^6$  a typical lesion should result.

We have compared the effect of inoculation on a range of specific-pathogen-free (SPF) inbred and non-inbred mice

**Fig. 1** a, AKR strain reaction to Lister vaccinia. b, NZB strain reaction to Lister vaccinia. c, A strain reaction to (i) Lister, (ii) LAC vaccinia. d, B10.D2 strain reaction to (i) Lister, (ii) LAC vaccinia.



**Table 1** An assessment of the reaction of inbred strains to two vaccinia vaccines

| Strain | Lister vaccine | LAC vaccine | Strain       | Lister vaccine | LAC vaccine | Strain   | Lister vaccine | LAC vaccine | Strain                  | Lister vaccine | LAC vaccine |
|--------|----------------|-------------|--------------|----------------|-------------|----------|----------------|-------------|-------------------------|----------------|-------------|
| A      | ++++           | ++++        | C57L         | ++++           | ++          | CBA/Ca   | ++++           | +++         | CBA/Y*                  | +++            | ND          |
| AKR    | ++++           | +           | LACG         | ++++           | +           | NZB      | +              | 0           | B10.Y*                  | +++            | ND          |
| A2G    | ++++           | +           | C57BL        | ++++           | ++++        | +/nu     | ++/+           | 0/+         | HTT*                    | +++            | ND          |
| NMRI   | +++            | ++          | C57BR        | ++++           | ++          | 129      | +++            | 0           | AKM*                    | +++            | ND          |
| BALB/c | +++            | +/+         | C57BL/10ScSn | ++++           | ++++        | NIH      | +++            | ND          | 4R×5R(F <sub>1</sub> )* | +++            | ND          |
| LACA   | ++++           | ++++        | B10.A        | ++++           | 0/+         | CBAT6*   | ++++           | ND          | 4R*                     | ++             | ND          |
| ICFW   | +++            | +           | B10.BR       | ++             | +           | B10A×4R* | +++            | ND          | 5R*                     | +++            | ND          |
| NZW    | +++            | +           | B10.D2       | +++            | +           | CSI*     | +++            | ND          | —                       | —              | —           |
| CE     | +++            | 5×0/+       | B10.LP-a     | +++            | 0           | CRH*     | +++            | ND          | —                       | —              | —           |
| DBA/1  | +++            | 0/+         | C3H/He-mg    | ++++           | +           | A/Jax*   | +++            | ND          | —                       | —              | —           |
| DBA/2  | +++            | +++         | C3H/He       | +++            | +           | PY*      | +++            | ND          | —                       | —              | —           |

ND, not done; 0, negative; +, positive. Grades of reaction represented by number of plus signs. Mixed reaction represented by subscript plus. \*Groups of three mice only.

known to be free of ectromelia. A total of 24 different strains maintained within the SPF unit of the LAC were tested for their response to the Lister strain of vaccinia and also to a weak strain of the virus donated to the LAC 4–5 yr previously. As the strain of this virus is not definitely known, it will be referred to in this paper as the 'LAC' strain. Observations were also made on other strains of mice sent for screening on which only the Lister vaccine was used.

The virus antigen used for screening was freeze-dried small-pox vaccine (Lister Institute) in 25 dose ampoules for human use, and had approximately  $1.0 \times 10^9$  PFU ml<sup>-1</sup>. The weak LAC strain originating from a human vesicle was passaged four times through eggs and assessed as being less than  $1.0 \times 10^2$  PFU ml<sup>-1</sup>. Groups of 6 SPF mice between 6 and 8 weeks of age from each of the following strains were used for testing both Lister and LAC vaccine: A, A<sub>2</sub>G, AKR, B10.A, B10.D2, B10.LP-a, BALB/c, C3H/He, C3H/He-mg, C57BL/10ScSn, C57BL, C57BR, C57L, CBA/Ca, CE, DBA/1, DBA/2, ICFW, LACA, LACG, NMRI, +/nu, NZB. Other strains, using the Lister vaccine only, were: 129, AKM, B10.A(4R), B10.Y, CBA/H-T6, CBA/Y, CRH, CSI, HTT, NIH, B105R, PY. All groups of mice, whether from the SPF unit or sent from outside for screening, were placed in PVC boxes fitted with a filter lid made from sterilised FG50 glass fibre filter material. After inoculation, the groups were maintained in similar filter containers until tested.

A small area of the dorsal surface at the base of the tail was carefully scraped with a sterile scalpel until the upper epidermal layers were removed and blood was drawn. A microdrop of the virus antigen was placed on the scarified area, and the test read 8–10 d later. In a positive reactor, the site of inoculation was usually swollen with either a raised pinkish cream blister or had a reddish focal area in the centre of the oedematous tissue. In non-reactors the site of scarification had completely healed and returned to normal.

Results obtained from the majority of mice inoculated with Lister vaccinia vaccine were good, and those of the LAC vaccine variable (Table 1). A poor response to the Lister strain was noted in only one of the coloured strains, the NZB (Fig. 1b); a mixed response in five groups, the B10.D2 and +/nu, B10.A(4R), CSI and CRH. All albino strains responded very well to the Lister vaccine with very little variation and Fig. 1a shows a typical example of a good reaction in AKR strain. The coloured strains varied in response slightly but all were recognisably positive (Table 1). Strains A, DBA/2 and C57BL/10ScSn reacted well to the LAC vaccine, as did LACA, C57BL, CBA/Ca. The NMRI and ICFW strains, on the other hand, showed a reduced reaction, the C3H/He-mg produced a very weak response and the B10.D2, NZB and the 129 strains were all negative (Fig. 1c and d). Variation in response to the Lister vaccine by individuals within a strain was on the whole very limited. Some B10.D2 and +/nu did not give a marked response, but all were clearly positive. In all other strains the six mice in each group tended to show a uniform reaction.

Despite the very low PFU of the LAC vaccine, reactions equivalent to the Lister vaccine occurred in six of the strains tested—A, LACA, DBA/2, C57BL, C57BL/10ScSn and CBA/Ca. Three strains showed marked variation—the +/nu, DBA/1 and B10A—which all had mixed positive and negative reactions. Three strains were clear negatives—B10.D2, NZB and 129. All other strains, although eliciting a weaker reaction than with the Lister strain, gave clear positive reactions. Thirteen other strains were tested with the Lister vaccine. Fewer mice were available for testing, but each gave very good positive reactions (Table 1).

The tail scarification method seems to be a very useful method of screening, provided it is carefully carried out. It does not seem likely that false negatives would be found using the Lister vaccine with its high PFU. Of 37 strains tested all produced a recognisably positive result, and it is fairly easy to read even in poor reactors, as the blisters, however small, are unmistakable. It is important, however, that wherever possible the same operator should perform the scarification and application of the antigen, to ensure uniformity of technique. The importance of uniformity in technique was indicated by the tests run on the CE strain. As a result of a change of technician, irregular results were obtained from both vaccinated groups. The test was rerun and uniform results recorded from both groups. Also, suitable controls are necessary when testing inbred strains. Where possible, SPF mice of the same strain should be used as controls whenever suspect colonies are to be screened.

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<sup>1</sup> Briody, B. A., *Bact. Rev.*, 23, 61–95 (1959).

## Mitigation of virus-induced foetal growth retardation in mice by dietary casein hydrolysate

EXPERIMENTAL infection of pregnant mice with Coxsackievirus B3 induces retarded foetal growth and development of the plasma proteins<sup>1,2</sup>. Subsequent work in mice indicated that the clinically mild illness induced by the virus in the mother may be largely responsible for the impaired foetal development, even though a direct effect of the virus in the foetus could not be ruled



**Table 1** Influence of dietary casein hydrolysate supplement on foetal development in mice infected with Cocksackievirus B3

| Days of pregnancy when casein hydrolysate is given in diet | Treatment                                        | No. of animals | Maternal weight (g) | Total no. of live foetuses | Total no. of resorptions | Foetal weight (g) | Foetal plasma Alb/AFP |
|------------------------------------------------------------|--------------------------------------------------|----------------|---------------------|----------------------------|--------------------------|-------------------|-----------------------|
| 1-18                                                       | Live Cocksackievirus B3 + 10% casein hydrolysate | 10             | 40.200 ± 1.520      | 75                         | 13                       | 1.143*            | 0.761†                |
|                                                            | Live Cocksackievirus B3                          | 16             | 31.987 ± 1.057      | 52                         | 39                       | 0.806             | 0.526                 |
|                                                            | Heat-killed Cocksackievirus B3                   | 8              | 45.926 ± 1.823      | 80                         | 0                        | 1.248             | 0.807                 |
| 10-18                                                      | Live Cocksackievirus B3 + 10% casein hydrolysate | 15             | 38.251 ± 1.448      | 109                        | 13                       | 1.194             | 0.792                 |
|                                                            | Live Cocksackievirus B3                          | 11             | 36.742 ± 1.700      | 58                         | 21                       | 0.838             | 0.565                 |
|                                                            | Heat-killed Cocksackievirus B3                   | 9              | 47.990 ± 1.600      | 89                         | 3                        | 1.400             | 0.946                 |

\* Standard error: foetal weight ±0.172.

† Standard error: Alb/AFP ratio ±0.103.

out<sup>3</sup>. The infection resulted in maternal pancreatitis within 2 d, with the animals being incapable of digesting adequate amounts of important dietary constituents, notably proteins, to maintain normal foetal growth<sup>4</sup>, in spite of a higher intake of food by the infected mothers. We concluded that the pancreatic lesions resulted in infected animals being deprived of essential dietary proteins, and therefore determined whether the effects of the pathogen could be mitigated or perhaps overcome by the administration of a readily assimilable diet.

Pregnant mice (20-25 g on day 1 of pregnancy) were fed a powdered diet for breeding rodents supplemented with 10% w/w casein hydrolysate (Sigma, St Louis). On day 8 of pregnancy 0.3 ml of a suspension of Cocksackievirus B3 containing  $10^{5.85}$  ml<sup>-1</sup> of TCID<sub>50</sub> was given intramuscularly. A second group of mice, which received no dietary supplement, was also injected with the same virus suspension.

Table 1 shows that the foetuses from the supplemented group at day 18 of gestation, when the experiment was terminated, did not differ significantly in weight from the controls given heat-killed virus suspension, whereas those which had no supplement were significantly lower in weight ( $P < 0.01$ ).

In a second series of experiments dietary supplement was not given until 2 d after the virus suspension was administered. The group of mice which had received the supplement was significantly heavier (Table 1) than those which were given live virus alone ( $P < 0.01$ ), but similar in weight to the controls which received heat-killed virus.

The plasma protein pattern from each group of foetuses was examined by two-dimensional immunoelectrophoresis. Groups which received virus alone had a low albumin/ $\alpha_1$ -foetoprotein ratio (Alb/AFP), which has been shown previously to be associated with retarded foetal development<sup>1,2</sup>. The Alb/AFP ratio of foetuses from virus-infected mice receiving a diet supplemented with protein hydrolysate was only slightly lower than that of the control group given heat-killed virus (difference not statistically significant). The degree of mitigation was independent of the time when feeding of the dietary supplement began.

These results indicate that supplementation of the diet of pregnant mice infected with Cocksackievirus B3, by the addition of suitable quantities of simple peptides and amino acids in the form of protein hydrolysate, can mitigate the effects of the virus on foetal growth retardation. It seems that the dietary supplementation compensates for foetal protein deficiency arising as a consequence of the virus-induced destruction of maternal pancreatic exocrine tissue. Immunoelectrophoretic studies of foetal plasma proteins show that this mitigation is not merely limited to increasing the weight of the foetus but also extends to annulling the effects of the virus on the development of the normal plasma protein patterns.

Although the results of these studies apply to Cocksackievirus B3 infection, it seems possible that they may also apply to other infections which adversely affect foetal growth indirectly by their pathological effects on maternal organs necessary for optimal foetal development.

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<sup>1</sup> Coid, C. R., and Ramsden, D. B., *Nature*, **241**, 460 (1973).

<sup>2</sup> Coid, C. R., Ramsden, D. B., and Healy, M. R. J., *Med. Microbiol. Immun.*, **159**, 285 (1974).

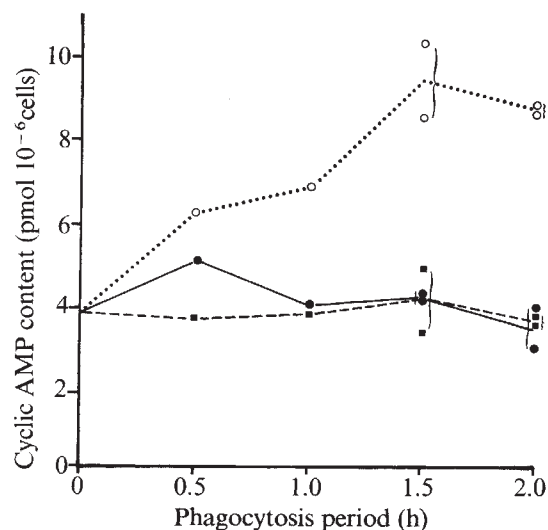
<sup>3</sup> Lansdown, A. B. G., and Coid, C. R., *Br. J. exp. Pathol.*, **55**, 101 (1974).

<sup>4</sup> Lansdown, A. B. G., and Ellaby, S. J., *Histochemistry*, **40**, 175 (1974).

## ***Mycobacterium microti* may protect itself from intracellular destruction by releasing cyclic AMP into phagosomes**

PATHOGENIC mycobacteria are able to survive and multiply within macrophages. How they do this is not known, but recent quantitative electron microscope surveys of intracellular events in macrophages infected *in vitro* with either *Mycobacterium tuberculosis* or *M. microti* revealed a lack of discharge of lysosomes into phagosomes containing live bacilli, whereas dead bacilli were associated with discharged lysosomal contents within phagolysosomes<sup>1,2</sup>. Possibly the living bacteria produce a factor which inhibits fusion between phagosomal and lysosomal membranes and thereby prevent the discharge of putatively bactericidal lysosomal contents into the bacterial environment. Since high intracellular levels of cyclic adenosine 3',5'-monophosphate (cyclic AMP) mediate pharmacological inhibition of lysosomal discharge in leukocytes<sup>3,4</sup> we reasoned that increased amounts of this nucleotide may occur in macrophages infected with living mycobacteria but not in those infected with dead ones. We report here experiments with *M. microti* showing that this is so and suggesting a bacterial origin for the additional cyclic AMP.

Marked elevation of the cyclic AMP content was observed only in monolayers ingesting live bacilli (Fig. 1). Minor disparities in the rates of association of the live and heat-killed bacteria with the macrophages as detected by light microscopy could not account for the difference. The increase in cyclic AMP correlated ( $P < 0.01$ ) with the number of cell-associated live bacilli (Fig. 2) and was remarkable since in other studies human polymorphonuclear leukocytes showed no change in cyclic AMP content during phagocytosis of latex spheres<sup>9,10</sup> or zymosan<sup>11</sup>. Furthermore, macrophages ingesting either bacilli killed by ultraviolet irradiation, latex spheres, colloidal gold or *M. lepraemurium* showed little or no increase in cyclic



**Fig. 1** Macrophage cyclic AMP content during phagocytosis. Macrophage monolayers were obtained from CFLP mice (Anglia Alconbury, UK) and 6–13 d later exposed to bacteria and other ingestible particles as described previously<sup>1</sup> except that the cells were maintained in plastic Petri dishes (diameter 5 cm) with about  $1.0 \times 10^6$  cells per dish. *M. microti* was grown in a chemostat in an asparagine-casamino acid medium (Dubos Broth Base,  $\times 4$  concentration; Difco) at a dilution rate of  $D = 0.016 \text{ h}^{-1}$  and about  $8 \times 10^8$  organisms  $\text{ml}^{-1}$ . Samples were centrifuged, resuspended in infection medium (2.5% horse serum in Hanks' balanced salt solution (BSS)) to yield  $1-8 \times 10^8$  bacilli  $\text{ml}^{-1}$ , essentially free of clumps and 100% viable. BSS-rinsed monolayers were overlaid with the suspension at a bacteria-cell ratio of 300–2,200:1 for 2 h at 37 °C. Monolayers of  $8.4 \times 10^6$  macrophages were incubated with 4 ml infection medium either alone (■) or containing  $2.0 \times 10^8$  live (○) or heat-killed (●) *M. microti*  $\text{ml}^{-1}$ . At 0.5 h intervals monolayers were chilled on ice, rinsed twice with ice-cold BSS and the majority of the cells scraped free into 2 ml BSS. After adding 1 ml 12% (v/v) perchloric acid they were briefly ultrasonicated and duplicate aliquots assayed for DNA (ref. 5) and cyclic AMP. Cyclic AMP was assayed using a competitive protein binding method<sup>6</sup> after initially purifying neutralised aliquots on AG50W- $\times 8 \text{ H}^+$  resin by washing with 1 mM potassium phosphate buffer, pH 7.0, and eluting with water<sup>7</sup>. The number of macrophages per monolayer was calculated from the DNA recovered ( $28.6 \mu\text{g} = 10^6$  cells). Since bacterial DNA contributed significantly to the total DNA in monolayers exposed to bacteria, the mean of the DNA contents of the resting (infection medium alone) monolayers was used throughout for calculating the cyclic AMP-cell ratios. Typically, the coefficient of variation of the resting cell DNA values was about 12%. The rates of phagocytosis of living and of dead bacteria were similar, as estimated by light microscopy of the residual monolayer cells stained using Kinyoun's modification of the Ziehl-Neelsen stain<sup>8</sup>. Random fields were examined until a total of 200 cells had been counted, the numbers of cells containing 0, 1–10, 11–20, 21–50 or > 50 bacilli were recorded and the mean number of bacilli per cell calculated assuming 0, 5, 15, 35 and 60 per cell in each respective score group.

AMP (Table 1). The lack of response with *M. lepraemurium*, in spite of an average of up to 12 cell-associated bacilli per cell, is consistent with the lysosome-phagosome fusion observed with this organism<sup>2</sup>: its success as an intracellular parasite may result from a special lipid coat protecting against inimical lysosome contents<sup>12</sup>.

That the elevation of cyclic AMP in monolayers exposed to live *M. microti* was not caused by the cellular biochemical events of phagocytosis was suggested by experiments in which monolayers were thoroughly rinsed after a phagocytosis period of 2 h and incubated for a further 2 or 4 d in maintenance medium<sup>1</sup> before assay. Increased levels of cyclic AMP were still apparent (Table 1). In this instance the lysosomes were labelled with colloidal gold before phagocytosis of mycobacteria so that lysosome-phagosome fusion could be assessed by electron microscopy<sup>1</sup> of monolayers treated identically to

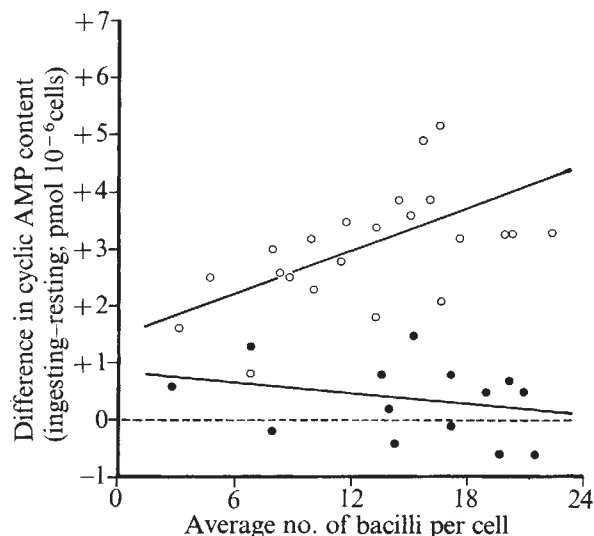
those assayed for cyclic AMP. Phagolysosome formation, as revealed by electron-dense gold particles in the same vesicles as bacilli, was detected only in cells exposed to dead organisms.

Although a contribution from macrophage metabolism has not been discounted, the additional cyclic AMP in *M. microti* infected cells could have originated from the bacteria as shown by measurement of their cyclic AMP content and generating capacity. Typically,  $2 \times 10^7$  bacteria from the chemostat contained 0.24 pmol; this was not changed significantly after 2 h at 37 °C in infection medium but it was lost after heat-killing. Thus, if their cyclic AMP content was unaltered during the infection process, 20 live bacteria per cell would have contributed 0.24 pmol to  $10^6$  macrophages. This is inadequate to account for the 4 pmol increase observed (Fig. 2). Allowance must be made for possible underestimation of bacterial numbers by light microscopy with heavily infected cells and for possible increase in the rate of bacterial cyclic AMP synthesis within phagosomes. Preliminary studies revealed that the environment of the bacillus can affect cyclic AMP synthesis sufficiently to remove the discrepancy:  $2 \times 10^7$  organisms produced 4 pmol cyclic AMP within 1.5 h at 37 °C in phosphate buffered saline (Dulbecco A; Oxoid, London).

*M. microti* inefficiently retained the synthesised nucleotide; in the chemostat 80% was extracellular and in phosphate buffer 88% was released within 3.5 h. The bacillus clearly possesses potential for liberating cyclic AMP into phagosomes in amounts which may inhibit lysosomal fusion. Mammalian cells are relatively impermeable to exogenous cyclic AMP (ref. 13). If the 3–7 pmol cyclic AMP increment detected 4 d after infection (Table 1) was produced by the bacilli residing in phagosomes of  $1.5 \times 10^{-13}$  (the approximate bacterial volume) impermeable to cyclic AMP an intraphagosomal concentration of about  $7 \times 10^{-5} \text{ M}$  could be inferred from the number of bacteria present. This is similar to the  $10^{-4} \text{ M}$  extracellular cyclic AMP which inhibited macrophage lysosome fusion<sup>3</sup>.

Other successful intracellular pathogens which are able to prevent lysosome-phagosome fusion are *Toxoplasma gondii*<sup>14</sup>, chlamydia<sup>15</sup> and perhaps *Neisseria gonorrhoeae*<sup>16</sup>. Measurements of cyclic AMP levels in cells infected with these agents

**Fig. 2** Dependence of macrophage cyclic AMP content on degree of infection. Data were pooled from five similar independent experiments in which levels of cyclic AMP were determined in macrophages which had been either resting or ingesting live or heat-killed *M. microti* for up to 2 h. Symbols and experimental details as in Fig. 1 except that the difference in cyclic AMP content between ingesting and resting cells is plotted and the number of macrophages per monolayer and the dose of bacteria varied between experiments. Regression of  $y$  on  $x$  was fitted by the least squares method.





**Table 1** Cyclic AMP content of macrophage monolayers exposed to a range of ingestible particles

| Treatment                                              | Experiment 1<br>after contact with<br>particles for: |             | Experiment 2<br>after contact with<br>particles for: |                   | Experiment 3<br>exposed to particles*<br>and then maintained<br>for a further: |                     |
|--------------------------------------------------------|------------------------------------------------------|-------------|------------------------------------------------------|-------------------|--------------------------------------------------------------------------------|---------------------|
|                                                        | 1 h                                                  | 2 h         | 1 h                                                  | 2 h               | 2 d                                                                            | 4 d                 |
| Control                                                | 1.7<br>1.4                                           | 1.7<br>2.1  | 2.4<br>2.7                                           | 3.0<br>2.8        | 3.7<br>4.8                                                                     | 6.1<br>5.7          |
| Live <i>M. microti</i> †                               | 4.9<br>4.0                                           | 5.0<br>4.5  | 5.0<br>5.7<br>5.8                                    | 6.9<br>6.7<br>6.3 | 7.4<br>7.1<br>8.0                                                              | 10.8<br>9.4<br>12.6 |
| Heat-killed<br><i>M. microti</i> †                     | —<br>—<br>—                                          | —<br>—<br>— | —<br>—<br>—                                          | —<br>—<br>—       | 3.9<br>3.6<br>—                                                                | 5.2<br>4.3<br>6.2   |
| Lethally ultraviolet<br>irradiated <i>M. microti</i> † | 2.8<br>2.2                                           | 2.6<br>2.2  | —<br>—                                               | —<br>—            | —<br>—                                                                         | —<br>—              |
| Latex spheres‡                                         | 2.3<br>1.7                                           | 1.5<br>2.3  | —<br>—                                               | —<br>—            | —<br>—                                                                         | —<br>—              |
| <i>M. lepraemurium</i> §                               | 2.9<br>2.4                                           | 2.3<br>2.6  | —<br>—                                               | —<br>—            | —<br>—                                                                         | —<br>—              |
| Colloidal gold¶                                        | —<br>—<br>—                                          | —<br>—<br>— | 3.1<br>3.2<br>2.7                                    | 3.0<br>2.1<br>2.1 | —<br>—<br>—                                                                    | —<br>—<br>—         |

Measurements on individual monolayers are shown.

\*All monolayers in Experiment 3 were treated first with 2.5 ml of colloidal gold (particles of up to 20 nm diameter; Radiochemical Centre, Amersham) which had been diluted to 500 µg ml<sup>-1</sup> in infection medium.

†The suspension of *M. microti* used presented a bacteria-cell ratio of 1,200, 2,200 and 500:1 in experiments 1, 2 and 3, respectively.

‡A suspension of microspheres with a mean diameter of 500 nm (Polaron Equipment, Watford, UK) was diluted in infection medium and used at a particle-cell ratio of 1,900:1.

§*M. lepraemurium* (Dr R. J. W. Rees) was a partially purified suspension obtained from mouse spleen and containing  $6.6 \times 10^{11}$  bacilli ml<sup>-1</sup> in 0.1% (w/v) bovine serum albumin saline. It was diluted with infection medium and used at a bacteria-cell ratio of  $1.2 \times 10^6$ :1.

¶Obtained and used as described above\*.

Other experimental details were as described in Fig. 1 and in the text.

might reveal the nucleotide as a common mediator of the infection-induced paralysis of intracellular digestion.

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- Armstrong, J. A., and Hart, P. D'A., *J. exp. Med.*, **134**, 713-740 (1971).
- Hart, P. D'Arcy, Armstrong, J. A., Brown, C. A., and Draper, P., *Infect. Immunity*, **5**, 803-807 (1972).
- Weissmann, G., Zurier, R. B., and Hoffstein, S., *Am. J. Path.*, **68**, 539-559 (1972).
- Zurier, R. B., Weissmann, G., Hoffstein, S., Kammerman, S., and Tai, H. H., *J. clin. Invest.*, **53**, 297-309 (1974).
- Giles, K. W., and Myers, A., *Nature*, **206**, 93 (1965).
- Brown, B. L., Albano, J. D. M., Ekins, R. P., Spherzi, A. M., and Tampion, W., *Biochem. J.*, **121**, 561-562 (1971).
- Kuo, J. F., and Greengard, P., *Adv. Cyclic Nucleotide Res.*, **2**, 41-50 (1972).
- Kinyoun, J. J., *Am. J. publ. Hlth*, **5**, 867 (1915).
- Stossel, T. P., Murad, F., Mason, R. J., and Vaughan, M., *J. biol. Chem.*, **245**, 6228-6234 (1970).
- Stolc, V., *Biochim. biophys. Acta*, **264**, 285-288 (1972).
- Ignarro, L. J., and George, W. J., *J. exp. Med.*, **140**, 225-238 (1974).
- Draper, P., and Rees, R. J. W., *Nature*, **228**, 860-861 (1970).
- Szabo, M., and Burke, G., *Biochim. biophys. Acta*, **264**, 289-299 (1972).
- Jones, T. C., and Hirsch, J. G., *J. exp. Med.*, **136**, 1173-1194 (1972).
- Friis, R. R., *J. Bact.*, **110**, 706-721 (1972).
- Novotny, P., Short, A. L., and Turner, W. H., *Proc. Soc. gen. Microbiol.*, **2**, 18 (1974).

## Spontaneous progressive multifocal leukoencephalopathy (PML) in macaques

PROGRESSIVE multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system (CNS) which has so far been recognised only in man<sup>1,2</sup>. Although it is an infrequent cause of clinical neurological disorder<sup>3</sup>, it is of particular interest because of its frequent association

with diseases in which immunological competence is impaired<sup>4-7</sup>, and because it is the only chronic demyelinating disease of man in which a virus (papovavirus) has been consistently demonstrated<sup>4,5,8,9</sup>. So far, efforts to transmit the disease to non-human primates have failed<sup>8</sup>. This report of a spontaneous disease in macaques with striking similarities to PML seems to be the first recorded instance of such a condition in animals.

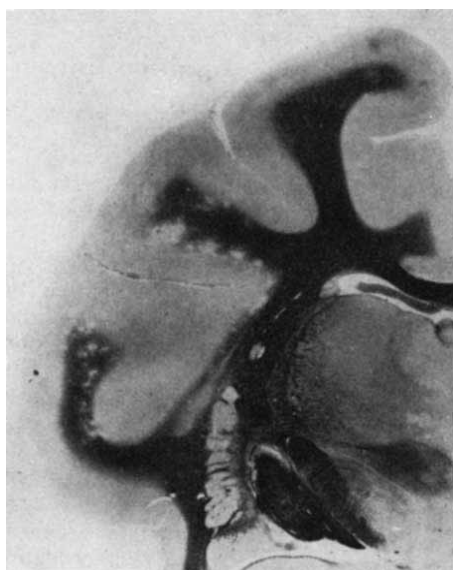
While reviewing the histology of approximately 400 monkey brains collected at autopsy, we found eight macaques (seven *Macaca mulatta* and one *M. speciosa*) with lesions similar to those described for PML. Electron microscopic examination of formalin-fixed brain tissue revealed papova-like virions in six of these monkeys.

A precise age (Table 1) was known for the two animals born in captivity. The remaining six were captured in the wild and had been at this centre 23-64 months; all were adults estimated to be over 8 yr old. The disparity in sex—seven females and one male—is a reflection of sex distribution of monkeys of this age in the colony. All monkeys received isoniazid in their diet while at the centre as prophylaxis for tuberculosis (227 mg isoniazid and 11.37 mg pyridoxine per pound of food).

Macroscopic lesions consisting of soft translucent foci were recognised when slices of fixed brain tissue were examined closely. These foci were usually 1-2 mm wide, and were randomly located, mainly in the subcortical white matter (Fig. 1), hypothalamus, medulla oblongata and adjacent to the cerebral aqueduct. A firm nodule, 1 cm in diameter, was present in the basilar meninges and compressed the adjacent brain in case 7. In case 6, the left optic nerve was encased by a layer of fibrous tissue which extended to the eyeball.

Histological foci of demyelination were present in all brains. Larger demyelinated areas seemed to result from confluence of smaller foci. Most demyelinated foci encompassed or were immediately adjacent to blood vessels.

Microglia and large astrocytes (gemästete glia) were present in and adjacent to foci of demyelination. Some nuclei of the oligodendrocytes in these areas were enlarged. Large polygonal cells, judged to be of astrocytic origin, with eosinophilic fibrillar cytoplasm and large vesicular nuclei, were present among the cellular accumulations (Fig. 2). These cells often contained



**Fig. 1** Foci of demyelination typical of the changes present in monkeys with PML. (Luxol Fast Blue  $\times 2.3$ ).

multiple nuclei and on rare occasions mitotic figures. Similar focal cellular accumulations were present in the grey matter.

Basophilic and eosinophilic intranuclear inclusion bodies were present in oligodendroglia and astrocytes of all eight monkeys. Cells with inclusions were in or adjacent to lesions in the grey and white matter but varied considerably in frequency from case to case. Eosinophilic inclusions were usually separated from a ring of marginated chromatin whereas the basophilic inclusions filled the nucleus (Fig. 2).

Perivascular cuffs consisting of lymphocytes, monocytes and rarely plasma cells were present in all brains. In three cases the cuffs were prominent but in the other five they were minimal to moderate in extent.

The spinal cords of seven monkeys were examined. Lesions similar to those in the brain were present in four. The optic nerve and retina were similarly affected in two out of five monkeys.

The nodule in the basilar meninges of case 7 and the fibrous tissue surrounding the left optic nerve of case 6 were interpreted as unusual fibroproliferative lesions. A marked reactive gliosis was present in the neuropil adjacent to these meningeal lesions. A similar nodule approximately 0.5 mm in diameter was present in the left frontal lobe of case 5.

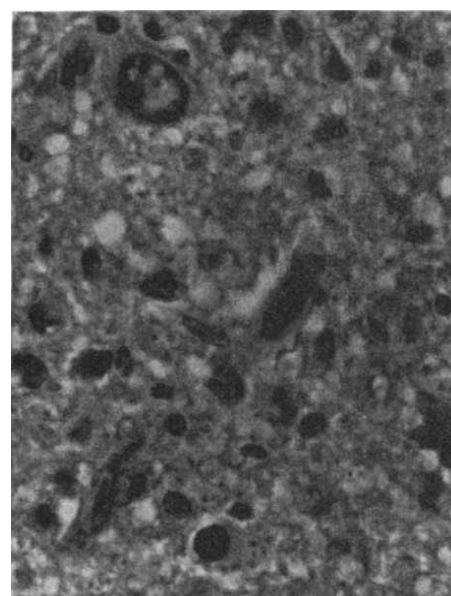
Electron microscopic examination was carried out on formalin-fixed brain tissue which had been processed in the usual fashion and embedded in Epon, or, on paraffin sections stained with haematoxylin and eosin which had been re-embedded in Epon<sup>10</sup>. Particles which were round, without envelopes and approximately 30 nm in diameter were found in

cells with inclusion bodies. Some particles were uniformly dense while others consisted of a dense peripheral ring (capsid) surrounding a lucent core. The particles were usually numerous and randomly distributed throughout the nucleus (Fig. 3). Occasionally large or small aggregates formed a lattice or crystalloid arrangement. Viral particles were rarely found in the cytoplasm and no filamentous forms have been observed. In our experience in monkeys, these particles are unique to the cases described here.

Most human cases of PML have occurred in elderly patients with lymphomas or chronic inflammatory processes<sup>1,2</sup>. Others have been associated with immunosuppressive therapy<sup>4,11</sup>.

The disease is characterised clinically by progressive neurological abnormalities lasting 3–12 months<sup>3</sup>. In rare cases patients have shown no neurological signs<sup>8</sup> or had no other concomitant disease processes<sup>12–14</sup>.

The histological changes are characterised by focal demyelination<sup>1,2</sup>. Large oligodendrocytes and astrocytes, some of the latter having been described as giant and bizarre, are



**Fig. 2** Photomicrograph from the periphery of a focus of demyelination demonstrating the typical cellular reaction. A giant cell and a basophilic nuclear inclusion body are present. (Haematoxylin and eosin  $\times 560$ ).

found in and adjacent to the demyelinated foci. Intranuclear inclusion bodies can usually be found in the oligodendroglia and less frequently in astroglia. An inflammatory cellular reaction is variable, being absent or modest in most cases but prominent in others.

While immunological competence was not determined in the monkeys reported here, observations in other monkeys having

**Table 1** Summary of spontaneous progressive multifocal leukoencephalopathy in eight macaques

| Case no. | Species            | Sex    | Age   | Neurological signs                                  | Concomitant disease(s)                                         | Severity of CNS lesions | Papova-like virions in glia |
|----------|--------------------|--------|-------|-----------------------------------------------------|----------------------------------------------------------------|-------------------------|-----------------------------|
| 1        | <i>M. mulatta</i>  | Male   | Adult |                                                     | Chronic haemolytic anaemia, mild giant cell pneumonia          | +                       | +                           |
| 2        | <i>M. mulatta</i>  | Female | Adult | Anisocoria, progressive rear-leg paralysis for 12 d | Moderate giant cell pneumonia                                  | +++                     | +                           |
| 3        | <i>M. speciosa</i> | Female | Adult |                                                     | Chronic colitis (shigellosis), mild giant cell pneumonia       | +++                     | +                           |
| 4        | <i>M. mulatta</i>  | Female | Adult |                                                     |                                                                | ++                      |                             |
| 5        | <i>M. mulatta</i>  | Female | Adult |                                                     | Lymphoma, Avian TB, chronic anaemia                            | ++                      |                             |
| 6        | <i>M. mulatta</i>  | Female | Adult |                                                     | Lymphoma, intestinal amyloidosis                               | ++                      | +                           |
| 7        | <i>M. mulatta</i>  | Female | 7 yr  | Lethargic                                           | Lymphoma, Avian TB                                             | ++                      | +                           |
| 8        | <i>M. mulatta</i>  | Female | 6 yr  | Ataxia for 5 d                                      | Chronic colitis (shigellosis), chronic vegetative endocarditis | ++                      |                             |



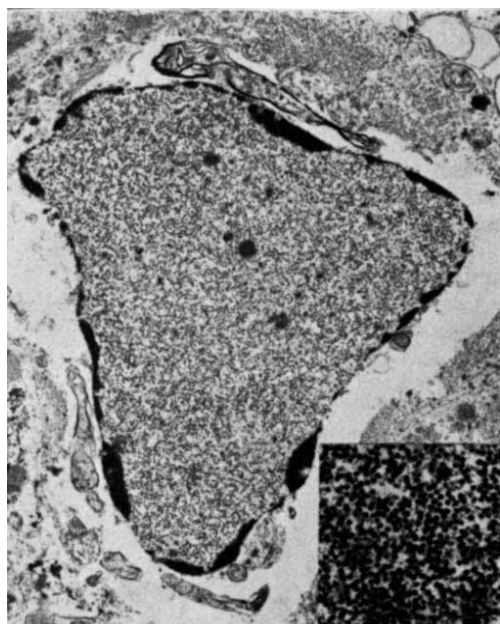


Fig. 3 Electron micrograph demonstrating papova-like virions in the nucleus of a glial cell. ( $\times 3,780$ , inset  $\times 17,955$ ).

spontaneous lymphoma or avian tuberculosis suggest impairment occurs (T. G. Terrell and C. A. Holmberg, personal communication). Isoniazid had been included daily in the diet of these monkeys and it has been suggested that this drug may impair immunological competence also<sup>15,16</sup>.

Jungherr *et al.*<sup>17</sup> examined the CNS of macaques experimentally infected with a papovavirus (SV40), and described non-suppurative inflammation. Hypertrophied, or 'pseudo-giant', ependymal cells were observed in some cases but no mention was made of inclusion bodies, or demyelination. All animals were killed on days 17–21 after inoculation.

We believe that the CNS disease described here in macaques has more similarities than differences to PML in man, and is a manifestation of infection by a papovavirus. Further search and evaluation of the factors causing the disease in monkeys may give insight into PML in man and other chronic viral infections of the CNS.

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- 1 Astrom, K.-E., Mancall, E. L., and Richardson, E. P., Jr., *Brain*, **81**, 93–111 (1958).
- 2 Richardson, E. P., Jr., *New Engl. J. Med.*, **265**, 815–823 (1961).
- 3 Weiner, L. P., Johnson, R. T., and Herndon, R. M., *New Engl. J. Med.*, **288**, 1103–1110 (1973).
- 4 Weiner, L. P., *et al.*, *New Engl. J. Med.*, **286**, 385–390 (1972).
- 5 Narayan, O., Penney, J. B., Jr., Johnson, R. T., Herndon, R. M., and Weiner, L. P., *New Engl. J. Med.*, **289**, 1278–1282 (1973).
- 6 Knight, A., O'Brien, P., and Osoba, D., *Ann. Intern. Med.*, **77**, 229–233 (1972).
- 7 Ellison, G. W., *J. Neuropath. exp. Neurol.*, **28**, 501–506 (1969).
- 8 Zuerlein, G. M., *Prog. med. Virol.*, **11**, 185–247 (1969).
- 9 Padgett, B. L., Walker, D. L., Zuerlein, G. M., Eckroade, R. J., and Dessel, B. H., *Lancet*, **i**, 1257–1260 (1971).
- 10 Pinkerton, H., and Carroll, S., *Am. J. Path.*, **65**, 543–548 (1971).
- 11 Manz, H. J., Dinsdale, H. B., and Morrill, P. A. F., *Ann. Intern. Med.*, **75**, 77–81 (1971).
- 12 Bolton, C. F., and Rozdilsky, B., *Neurology, Minneapolis*, **21**, 72–77 (1971).
- 13 Silverman, L., and Rubinstein, L. J., *Acta Neuropath.*, **5**, 215–224 (1965).
- 14 Fernald, J., Hardman, J. M., and Earle, K. M., *Neurology, Minneapolis*, **20**, 479–484 (1970).
- 15 Gibson, J. P., Rohovsky, M. W., and Newberne, J. W., *Lab. Animal Sci.*, **21**, 62–66 (1971).
- 16 Dove, J. T., Chaparas, S. D., and Hedrick, S. R., *Am. Rev. resp. Dis.*, **106**, 485–487 (1972).
- 17 Jungherr, E. L., Cabasso, V. J., and Stebbin, M., *J. Neuropath. exp. Neurol.*, **22**, 512–527 (1963).

## Helper effect of normal and irradiated thymus cells on transferred immunoglobulin production

THE cellular transfer of immunoglobulin (Ig) production in mice by various lymphoid cell preparations has been demonstrated previously by the use of allotype congenic mouse strains<sup>1,2</sup>. Lymphoid cell suspensions from one mouse line are injected intravenously into histocompatible but allotype-different, partner-strain mice without deliberate immunisation, and the resulting Ig production ascertained by donor allotype analysis of the sera taken at various times. We have used this transfer system to determine whether anti- $\mu$  pretreatment of lymphoid cells results in suppression of subsequent IgG production<sup>3</sup>, and in studies on the radiosensitivity of the B cell series (R.E.A., and N.L.W., unpublished). In analysing the results of Ig transfer in such experiments, it has become evident that the possible role of T cells in either augmenting or suppressing the Ig transfer by B cells must be determined, and here we present evidence that such T cell involvement can be demonstrated to varying degrees.

Immunoglobulin transfer between three pairs of allotype congenic strains was studied. BALB/c (Ig<sup>a</sup> allotype) and C57BL/6 (Ig<sup>b</sup>) mice were used as recipients for spleen cell transfers from the respective donor strains BAB-14 (Ig<sup>b</sup> allotype), (Herzenberg), BALB/c. Ig<sup>b</sup> N.12 (Ig<sup>b</sup> allotype), and C57BL/6. Ig<sup>c</sup> (Ig<sup>c</sup> allotype) (both Hall Institute). All donor mice were homozygous at Ig loci and were derived by inbreeding after 14, 12 and 6 backcross generations, respectively. Single cell suspensions of donor spleens were prepared from 6–10 week old mice by teasing spleens through stainless steel sieves into 10% foetal calf serum in MEM. All cell transfers were made intravenously to groups of five to eight recipient mice 6–10 weeks of age, that had received 400 rad whole-body irradiation within the previous 24 h. Recipient mice were bled at various intervals and the levels of donor type IgG<sub>2</sub> allotype quantitated by radioimmunoassay<sup>3</sup>. All results are expressed as the amount of donor type IgG as a percentage of a standard

Fig. 1 Effect of added thymus cells on Ig transfer by allotype congenic spleen cells. Donor spleen cells— $4 \times 10^6$  C57BL/6.Ig<sup>c</sup>. All thymus cells added at 1:1 ratio. ●, Non-irradiated 7 week old thymus cells; ○, 1,000 rad 7 week thymus cells; △, Non-irradiated 7 month thymus cells. Amount of donor type (Ig<sup>c</sup>) Ig was assayed 1, 2 and 4 weeks after transfer and the relative amount of Ig in recipients of added thymus cells expressed relative to the amount with spleen transfer alone. Vertical bars and points show mean  $\pm$  s.e.

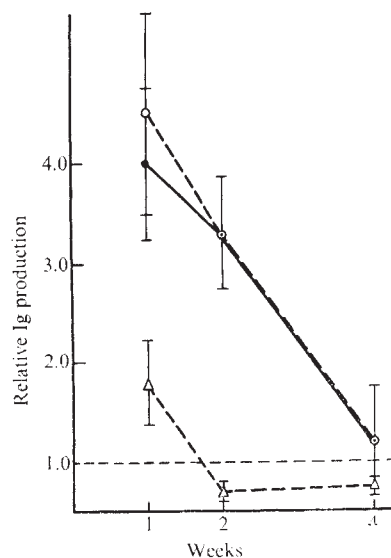


Table 1 Effect of T-cell depletion on Ig transfer

| Experiment no. | Donor                                      | Transfer conditions<br>Recipient | No. of spleen cells | Assay time<br>(d) | Percentage donor Ig<br>(mean $\pm$ s.e.) |
|----------------|--------------------------------------------|----------------------------------|---------------------|-------------------|------------------------------------------|
| 1              | BABB                                       | BALB/c                           | $7 \times 10^6$     | 7                 | $1.7 \pm 0.3$                            |
|                | Anti- $\theta$ BABB                        | BALB/c                           | $7 \times 10^6$     | 7                 | $0.9 \pm 0.1$                            |
|                | BABB                                       | BALB/c                           | $7 \times 10^6$     | 21                | $5.4 \pm 1.2$                            |
|                | Anti- $\theta$ BABB                        | BALB/c                           | $7 \times 10^6$     | 21                | $4.6 \pm 1.0$                            |
| 2              | BABB                                       | BALB/c                           | $8 \times 10^6$     | 7                 | $1.2 \pm 0.2$                            |
|                | Anti- $\theta$ BABB                        | BALB/c                           | $8 \times 10^6$     | 7                 | $0.9 \pm 0.1$                            |
| 3              | C57BL.Ig <sup>e</sup>                      | C57BL                            | $4 \times 10^6$     | 28                | $36.8 \pm 6.7$                           |
|                | Anti- $\theta$ C57BL.Ig <sup>e</sup>       | C57BL                            | $4 \times 10^6$     | 28                | $48.6 \pm 7.5$                           |
| 4              | CBA <i>nu/nu</i>                           | 700 rad C57BL                    | $10 \times 10^6$    | 7                 | $3.1 \pm 0.3$                            |
| 5              | Anti- $\theta$ BALB/c Ig <sup>b</sup> .N12 | BALB/c                           | $5 \times 10^6$     | 14                | $23.2 \pm 2.0^*$                         |
|                | Anti- $\theta$ BALB/c Ig <sup>b</sup> .N12 | BALB/c <i>nu/nu</i>              | $5 \times 10^6$     | 14                | $15.6 \pm 4.1^\dagger$                   |

\*All mice (21 out of 21) showed donor type Ig levels in the range 10–40%.

†Mean donor level for all mice (17) includes ten mice with ratios in the range 9–40% and a group of seven mice all having <2% donor Ig. The mean value for the ten mice showing >9% donor Ig was  $25.8 \pm 4.7\%$ , that is, similar to that in the 21 out of 21 intact recipients.

adult serum pool of donor strain type. In experiments on the addition of thymus cells, cell suspensions were similarly prepared from thymuses of mice of various ages that were syngeneic to the recipient strain.

As most of the previous cell transfer experiments have involved whole spleen cell preparations (that is, T and B cells), transferred into sublethally irradiated but otherwise intact recipients (that is, the recipient also has T cells), we first determined the effect of eliminating T cells from the donor inoculum with anti- $\theta$  treatment. In three such experiments (Table 1, experiments 1–3), anti- $\theta$  treated spleen cells were still capable of donor type Ig production after transfer, although in one experiment, a 50% reduction in donor type Ig was observed in the 1 week sample. Similarly, the use of spleen cells from nude mouse donors (on CBA (Ig<sup>a</sup> allotype) background) transferred into lethally irradiated C57BL recipients also resulted in Ig production. As these four experiments all involved transfer into recipients possessing T cells the functional capabilities of which in this context may be radio-resistant, we performed one cell transfer experiment with anti- $\theta$  treated BALB/c. Ig<sup>b</sup> spleen cells into homozygous nude BALB/c (Ig<sup>a</sup> allotype) mice (of six backcross derivation from BALB/c, Holmes). Whereas 21 out of 21 normal BALB/c recipients had donor Ig levels between 10% and 40% 14 d later (mean 23.2), 7 out of 17 of the nude recipients failed to support any detectable donor Ig production, and the remaining 10 had similar values to the intact mice (range 9–40, mean 25.8%).

These experiments suggest that the level of Ig production by donor B cells is not markedly influenced by either the proportion of T to B cells present in donor spleen preparations, the possible subtypes of T cells present, or the absolute number of transferred T cells. These results, however, do not deny the possibility that subpopulations of B cells in the spleen may be relatively dependent on help from T cells, and that in this

system of total Ig transfer without deliberate antigen injection, the past environmental exposure of the donor animal may influence the proportion of these B subtypes in spleen, and, for example, in experiment 1, the population dependent on T cells may be present to a relatively greater degree. Resolution of this aspect is being approached by the use of defined B cell populations separated by the Los Alamos fluorescence-activated cell sorter.

The results obtained in experiment 5 with transfer of anti- $\theta$  cells into nude recipients suggest that some nude mice may be incapable of supporting Ig transfer whereas others can do so. It is generally considered that nude mice are devoid of T cells, although several recent studies<sup>4</sup>, (Gutman, G., personal communication) suggest that this may not always be the case, and varying numbers of T cells may be found in the lymphoid tissues. It may therefore be that this Ig transfer system has an absolute requirement for at least a minimal number of T cells (in either donor cells or host animal), and the 7 out of 17 nude recipients may represent this total T-deficient state. Apart from this latter possibility, the present conclusion from these studies is that a large part of the Ig transfer is T-independent.

We therefore investigated whether additional T cells would augment or suppress the Ig transfer. The basic system was to use added thymus cells of mouse strain syngeneic to the recipient (in both allotype and histocompatibility). Numerous experiments of this type were performed with attention to several variables: ratio of thymus to spleen cells, age of thymus donor, strain combination, time of assay post transfer, and effect of irradiation of the thymus cells.

The effect of additional thymus cells on Ig transfer ascertained at several times after transfer is shown in Fig. 1. Addition of 7-week-old thymus cells at a 1:1 ratio to spleen cells, and using either intact or irradiated (1,000 rad) thymus, resulted in a fourfold increase in donor Ig transfer at 7 d, declining to no difference at 4 weeks. Over this period the absolute amount

Table 2 Effects on transferred immunoglobulin production of thymus cell addition to spleen cells

| Experiment no. * | Donor type immunoglobulin (% standard†) |                   |                              | Relative‡ increase    |                        |
|------------------|-----------------------------------------|-------------------|------------------------------|-----------------------|------------------------|
|                  | Control                                 | With thymus cells | With irradiated thymus cells | Thymus 400/<br>thymus | Thymus 400/<br>control |
| 1                | $0.6 \pm 0.1$                           | $2.1 \pm 0.4$     | $5.5 \pm 1.4$                | 2.6                   | 9.2                    |
| 2                | $6.0 \pm 1.0$                           | $41.5 \pm 3.1$    | $36.1 \pm 5.1$               | 0.9                   | 6.0                    |
| 3                | $18.0 \pm 7.2$                          | $60.0 \pm 12.0$   | $61.0 \pm 10.0$              | 1.0                   | 3.3                    |
| 4                | $0.8 \pm 0.3$                           | $0.6 \pm 0.2$     | $1.4 \pm 0.2$                | 2.3                   | 1.8                    |
| 5                | —                                       | $18.5 \pm 4.8$    | $35.2 \pm 4.5$               | 1.9                   | —                      |
| 6                | $1.3 \pm 0.2$                           | $0.3 \pm 0.1$     | $4.8 \pm 0.8$                | 16.0                  | 3.7                    |

\*The conditions of transfer for each of the experiments in terms of several variables were as follows: 1, Spleen donor C57BL/6.Ig<sup>e</sup>, thymus donor 10 week C57BL, ratio thymus: spleen 1:2, serum assay d 7. 2, Spleen donor BABB-14, thymus donor 5 week BALB/c, ratio thymus: spleen 1:1, serum assay d 14. 3, Spleen donor C57BL/6.Ig<sup>e</sup>, thymus donor 7 week C57BL, ratio thymus to spleen 1:1, serum assay d 14. 4, As for 3 but 6 d serum assay. 5, Peyer's patch lymphoid cell donor BABB-14, thymus donor 5 week BALB/c, ratio thymus to spleen 2:1, serum assay d 13. 6, Spleen donor C57BL/6.Ig<sup>e</sup>, thymus donor 1 d old C57BL, ratio thymus to spleen 4:1, serum assay d 14. All recipients are syngeneic to thymus donor. Irradiated thymus cell suspensions all given 400 rad.

†The results are expressed as percentage donor spleen type adult serum pool  $\pm$  s.e. mean.

‡Ratio of donor type Ig levels for groups given spleen cells and irradiated thymus cells to either spleen cells alone or spleen and non-irradiated thymus.



of donor Ig was increasing in the control mice, thus indicating a more rapid production of Ig in the thymus-added group, but reaching the same final level. The addition of 7-month-old thymus cells had a less pronounced effect, indicating a possible change in the thymus cellular population at this age.

A summary of six experiments is given in Table 2 and experiment 3 is depicted in Fig. 1. The relative effect of added intact thymus cells assessed 1–2 weeks after transfer shows that in five experiments (control mice not available in number 5) augmentation of Ig transfer was achieved in three, with little change or suppression being observed in the other two. In all six experiments, however, addition of irradiated (400 rad) thymus cells gave marked augmentation. In four experiments, Ig production resulting from added irradiated thymus was greater than with added non-irradiated thymus.

Further studies on the cellular specificity of this effect are in progress, although at present little augmentation has been observed with added bone marrow cells. Two observations suggest that these results are indicative of alternative functions of subsets of T cells on the B cell system. First, addition of 7-month-old or newborn thymus had relatively little augmentative effect, also indicating that the results are not nonspecifically related to some filler cell function which might be contributed by any added cell; and second, the increased relative effect of irradiated thymus to intact thymus is consistent with the thesis that a subpopulation of T cells can suppress Ig transfer, that is, perhaps analogous to suppressor T cells as described in other systems<sup>5</sup>, and that this suppressor population is relatively radiosensitive in comparison to the helper T cell subset, an observation made previously<sup>6,7</sup>. Also, the effect of radiation in distinguishing help from suppression was most pronounced with the newborn thymus, perhaps inferring the presence of a relatively greater proportion of suppressors at this age.

These results may also infer that the transfer of Ig or antibody production by direct injection of marked thymus cells could be markedly dependent on the relative proportions of helper and suppressor T cells in the thymus cell suspension relative to the 1–2% of contaminating B cells found in most thymus cell preparations<sup>8</sup>.

The use of the cellular transfer system of allotypically marked immunoglobulin is now being assessed as a model system for the analysis of the ontogeny of suppressor T cells and also, with appropriate allotype congenic NZB mice, in the role of suppressor T cells in spontaneous autoimmune disease. The relatively minor effect of T-cell depletion on Ig transfer reinforces the previous suggestion<sup>2</sup> that inhibition of IgG transfer following anti- $\mu$  chain treatment is a result of inhibiting  $\mu$ -bearing cells which otherwise were destined to switch to IgG secretion. Further analysis of this latter point is being made by direct use of cells sorted on the basis of membrane-bound Ig.

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- <sup>3</sup> Herzenberg, L. A., and Warner, N. L., in *Regulation of Antibody Response* (edit. by Cinader, B.), 322–348 (C. C. Thomas, Illinois, 1968).
- <sup>4</sup> Loor, F., and Roelants, G. E., *Ann. N.Y. Acad. Sci.* (in the press).
- <sup>5</sup> Gershon, R. K., *Contemp. Topics in Immunobiol.*, 3, 1–40 (1974).
- <sup>6</sup> Tada, T., Taniguchi, M., and Okumura, K., *J. Immunol.*, 106, 1012–1018 (1971).
- <sup>7</sup> Rotter, U., and Trainin, N., *Cell. Immunol.*, 13, 76–86 (1974).
- <sup>8</sup> Warner, N. L., *Adv. Immunol.*, 19, 67–216 (1974).

## T-cell dependence of immune response to hepatitis B antigen in mice

THE presence of hepatitis B surface antigen (HB<sub>s</sub>Ag) in blood is a marker of infection with hepatitis B virus (HBV). Persistence of the antigen in the blood of 'carriers' is a manifestation of proliferation of HBV in liver cells and this carrier state can be associated with various types of liver disease<sup>1</sup>. In guinea pigs and chimpanzees, immunisation with HB<sub>s</sub>Ag in Freund's complete adjuvant (FCA) elicits a cutaneous delayed type hypersensitivity (DTH) response<sup>2,3</sup>. It is believed that integrity of the thymus-dependent cell-mediated immune system in man is necessary for resistance to HBV, and that deficiency of cell-mediated immunity could predispose to development of the carrier state<sup>4,5</sup>. We therefore investigated the contribution of thymus-derived cells to the immune response to HB<sub>s</sub>Ag in mice by immunisation of BALB/c mice, mice heterozygous for the nude mutant gene (*nu*/+), and congenitally athymic mice of the *nu*/*nu* strain, using purified HB<sub>s</sub>Ag.

From the plasma of an adult asymptomatic carrier (subtype adw), HB<sub>s</sub>Ag was isolated by a combination of isopycnic banding and rate sedimentation using CsCl gradients<sup>6</sup>. The purified HB<sub>s</sub>Ag was free of human serum components. A reduced and alkylated form of HB<sub>s</sub>Ag (RA-HB<sub>s</sub>Ag) was prepared by treating purified HB<sub>s</sub>Ag (0.5 mg ml<sup>-1</sup>) with an equal volume of 0.4 M 2-mercaptoethanol for 3 h at 37 °C followed by iodoacetamide in the dark at 4 °C for 1 h. Purified HB<sub>s</sub>Ag was labelled with <sup>125</sup>I (carrier-free, Radiochemical Centre, UK) using chloramine-T as an oxidising agent<sup>7</sup>; the specific activity was 27–35  $\mu$ Ci  $\mu$ g<sup>-1</sup>.

Four mice from each of the three groups (BALB/c, *nu*/+ and *nu*/*nu*) were injected subcutaneously in four sites adjacent to lymph nodes with 20.0  $\mu$ g HB<sub>s</sub>Ag in 0.4 ml FCA. The FCA (Difco) contained 0.5 mg ml<sup>-1</sup> of *Mycobacterium butyricum*, and was supplemented with 4 mg ml<sup>-1</sup> of *M. tuberculosis* (H 37 RA, Difco). In addition four mice from each group were injected similarly with 20.0  $\mu$ g RA-HB<sub>s</sub>Ag in 0.4 ml FCA. After 18 d all 24 mice received an intracutaneous injection of 10.0  $\mu$ g HB<sub>s</sub>Ag in saline in the left hind footpad as a challenge for a DTH response. The next day the mice were killed, bled by cardiac puncture, and the spleen was removed and placed in Eisen's balanced salt solution. The left hind footpad was amputated and placed in formalin for fixation before preparing sections for histological examination.

Serum was tested under code, for antibody to HB<sub>s</sub>Ag by a haemagglutination assay<sup>8</sup> and for HB<sub>s</sub>Ag by a solid-phase radioimmunoassay (AUSRIA<sup>TM</sup>)<sup>9</sup>. A suspension of  $1 \times 10^7$  single cells from each spleen was mixed with 1.0  $\mu$ g <sup>125</sup>I-HB<sub>s</sub>Ag and tested for antigen-binding lymphocytes (ABL)<sup>10</sup>. The slides were examined under code for ABL and scored as previously described<sup>11</sup>; cells with 5–25 grains above background were scored as lightly labelled, and those with more than 25 grains as heavily labelled. DTH reactions were assessed only microscopically, with histological sections of the footpads being examined under code. The basis for scoring the intensity of DTH reactions was the degree of infiltration of cells of mononuclear type; for example 1+ represented minimal and mostly perivascular infiltration, and 4+ represented massive and diffuse infiltration throughout the section. The results are shown in Table 1.

In BALB/c mice and in heterozygotes the native HB<sub>s</sub>Ag induced high titres of antibody to HB<sub>s</sub>Ag. Concurrent titrations on native sera and sera treated with 2-mercaptoethanol

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- <sup>1</sup> Klein, J., and Herzenberg, L. A., *Transplantation*, 5, 1484–1495 (1967).
- <sup>2</sup> Herrod, H. G., and Warner, N. L., *J. Immunol.*, 108, 1712–1717 (1972).

**Table 1** Immune response to HB<sub>s</sub>Ag and RA-HB<sub>s</sub>Ag in athymic *nu/nu*, heterozygote and normal BALB/c mice

| Groups of mice<br>(four per group) | Immunised with            | DTH response to HB <sub>s</sub> Ag* | Mean ABL per 1,000 lymphocytes† |      |        | Antibody to HB <sub>s</sub> Ag<br>(geometric mean titre)‡ |
|------------------------------------|---------------------------|-------------------------------------|---------------------------------|------|--------|-----------------------------------------------------------|
|                                    |                           |                                     | H                               | L    | Total‡ |                                                           |
| <i>nu/nu</i>                       | Native HB <sub>s</sub> Ag | —, —, +, 2+                         | 0.9                             | 1.9  | 2.8    | 0                                                         |
| <i>nu/nu</i>                       | RA-HB <sub>s</sub> Ag     | +, +, 2+, 3+                        | 0.2                             | 1.7  | 1.9    | 0                                                         |
| <i>nu/+</i>                        | Native HB <sub>s</sub> Ag | 2+, 2+, 3+, 3+                      | 5.5                             | 12.0 | 17.5   | 75                                                        |
| <i>nu/+</i>                        | RA-HB <sub>s</sub> Ag     | 2+, 3+, 4+, 4+                      | 2.3                             | 5.5  | 7.8    | 45                                                        |
| BALB/c (+/+)                       | Native HB <sub>s</sub> Ag | 2+, 4+, 4+, 4+                      | 3.9                             | 12.2 | 16.1   | 75                                                        |
| BALB/c (+/+)                       | RA-HB <sub>s</sub> Ag     | 3+, 4+, 4+, 4+                      | 2.9                             | 9.6  | 12.5   | 53                                                        |

\*Presence and degree of DTH response to HB<sub>s</sub>Ag were assessed by histological evidence of mononuclear cell infiltration 24 h after intracutaneous challenge; neutrophil infiltration was negligible in all sections.

†Antigen binding lymphocytes per 1,000 spleen lymphocytes; H, heavily binding and L, lightly binding (see text). As controls, counts of splenic ABL for HB<sub>s</sub>Ag in non-immunised *nu/nu*, *nu/+* and BALB/c mice were respectively 0.9, 2.6 and 1.6.

‡Differences were significant (*t* test) between group *nu/nu* and other groups for counts of ABL ( $P < 0.005$ ) and antibody ( $P < 0.02$ ).

gave comparable results, indicating that the class of antibody to HB<sub>s</sub>Ag was IgG. Moreover, there were high counts of ABL in the spleen, and a pronounced DTH reaction in the skin. Immunisation with RA-HB<sub>s</sub>Ag gave comparable results for antibody, ABL, and DTH. On the other hand, the immune response to HB<sub>s</sub>Ag and RA-HB<sub>s</sub>Ag in athymic *nu/nu* mice was weak or absent, as judged by no detectable antibody, minimally raised counts of ABL in the spleen, and lower DTH responses. Tests for HB<sub>s</sub>Ag in serum were uniformly negative.

The above findings indicate that in mice, HB<sub>s</sub>Ag elicits strong DTH responses, and is highly thymic-dependent with regard to requirement for T-cell help for a humoral antibody response. The presence in athymic *nu/nu* mice of ABL in the spleen is consistent with the interpretation<sup>12</sup> that such ABL are B cells. Although RA-HB<sub>s</sub>Ag induced only DTH but no humoral immune response in guinea pigs<sup>2</sup>, in mice it induced both types; this observation supports the finding<sup>13</sup> that the immune response to RA-HB<sub>s</sub>Ag varies in different species. Thus, it could be suggested that the carrier state for HB<sub>s</sub>Ag in man is akin to a state of tolerance consequent on failure of T-cell recognition of immunogenic components of this antigen.

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- Huang, S. N. *et al.*, *Human Path.*, **5**, 209 (1974).
- Vyas, G. N., Rao, K. R., and Ibrahim, A. B., *Science*, **178**, 1300 (1972).
- Ibrahim, A. B., Vyas, G. N., and Prince, A. M., *Clin. exp. Immun.*, **17**, 311 (1974).
- Dudley, F. J., Fox, R. A., and Sherlock, S., *Lancet*, **i**, 527 (1972).
- Ibrahim, A. B., Vyas, G. N., and Perkins, H. A., *Infect. Imm.* (in press).
- Vyas, G. N., Williams, E. W., Klaus, G. G. B., and Bond, H. E., *J. Immun.*, **108**, 1114 (1972).
- Ada, G. L., Nossal, G. J. V., and Pye, J., *Aust. J. exp. Biol. med. Sci.*, **42**, 295 (1964).
- Vyas, G. N., and Shulman, N. R., *Science*, **170**, 332 (1970).
- Ling, C. M., and Overby, L. R., *J. Immun.*, **109**, 203 (1972).
- Dwyer, J. M., and Mackay, I. R., *Clin. exp. Immun.*, **10**, 581 (1972).
- Roberts, I. M., Whittingham, S., and Mackay, I. R., *Lancet*, **ii**, 936 (1973).
- Dwyer, J. M., Mason, S., Warner, N. L., and Mackay, I. R., *Nature new Biol.*, **234**, 252 (1971).
- Imai, M. *et al.*, *J. Immun.*, **112**, 416 (1974).

## Human lymphocytes recognise mouse alloantigens

H-2 LD antigens<sup>1</sup> of the mouse major histocompatibility complex and the lymphocyte-activating determinants of the *Mls* (ref. 2) locus induce vigorous lymphocyte proliferation and enhance generation of cytotoxic effector cells in allogeneic combinations (ref. 3 and D. J. Schendel, and F. H. Bach, unpublished). These antigens do not serve well as targets for cell-mediated lympholysis (CML)—a function which in the mouse is performed by the classical H-2 determinants, the SD antigens<sup>4,5</sup>. A similar differential function of LD and SD antigens is found in human allograft reactions *in vitro*<sup>6</sup>.

Although the polymorphism of the LD antigens clearly is extensive, it is possible that the difference between the LD alleles of two animals of the same species is only a few amino acid substitutions and not enough to allow effective recognition by cytotoxic effector cells. It might be expected that with increasing phylogenetic distance there would be a greater difference between the LD alleles of two animals, such that effector cells sensitised against the LD antigens from another species might show positive cytotoxicity towards these antigens.

Using human lymphocytes immunised *in vitro* against mouse spleen cells, we confirmed that xenogeneic effector cells are specifically cytotoxic to target cells of the same H-2 type as the sensitising strain<sup>7,8</sup> and found no evidence of differential recognition of any non-H-2 target<sup>9</sup> (see also Fig. 1 and Table 1). In this report we describe the fine specificity of H-2 target antigens in xenogeneic combinations. The major conclusion is that in xenogeneic as in allogeneic combinations, cytotoxicity is mainly directed towards the H-2 SD antigens, whereas the LD antigens do not function as targets, or do so only poorly.

The H-2 complex is divided into four regions: K, I, S and D; genes of the K and D regions (H-2K and H-2D loci) determine the H-2 SD antigens; the strong LD antigens are products of genes in the I region. Human lymphocytes were sensitised to each of the four strains B10.A(4R), B10.A(2R), AQR, and B10.T(6R), abbreviated 4R, 2R, AQR, and 6R, respectively, and their cytotoxic potential was measured on target cells from each strain. The genetic constitution of these strains enables us to assay the importance of the K and D regions versus the I and S regions as targets for cytotoxic effector cells (Fig. 2). If the SD antigens of the K and D regions are the prime targets for xenogeneic as for allogeneic effector cells, and the LD antigens do not serve well in this capacity, we would expect that human lymphocytes sensitised to 4R and 2R are equally and highly cytotoxic to targets of these strains and give little or no killing on target cells from AQR and 6R; as a corollary, effectors sensitised to AQR or 6R would give equal and high killing on targets of these two strains and only low killing on targets from 4R or 2R. As Fig. 1 shows, this is clearly the case.

We have carried out nine experiments of this type. One was excluded because there was no cytotoxicity on any target, and



**Table 1** Importance of products of K+D against I+S regions of H-2 as targets for xenogeneic effector cells—summary of eight experiments

| Target cells* | Effectors sensitised to: | H-2 regions shared with target | Cytotoxicity† (%) |           | P‡     |
|---------------|--------------------------|--------------------------------|-------------------|-----------|--------|
|               |                          |                                | Median            | Range     |        |
| 1R or 2R§     | 4R                       | K, I-A, D                      | 11.55             | 2.7–29.6  | >0.05  |
|               | 1R or 2R§                | K, I, S, D                     | 11.25             | 2.7–44.4  |        |
|               | AQR                      | I, S                           | 3.1               | 2.1–16.2  | >0.05  |
|               | 6R                       | —                              | 1.7               | –0.4–20.0 |        |
|               | H                        | —                              | 0                 | 0         | 0.025  |
| AQR           | 6R                       | K, D                           | 13.4              | 3.8–47.5  | >0.05  |
|               | AQR                      | K, I, S, D                     | 12.3              | 3.8–43.8  |        |
|               | 1R or 2R§                | I, S                           | 1.2               | –6.8–31.1 | <0.025 |
|               | 4R                       | I-A                            | 3.1               | –5.8–44.3 | >0.05  |
|               | H                        | —                              | 0                 | 0         | >0.05  |

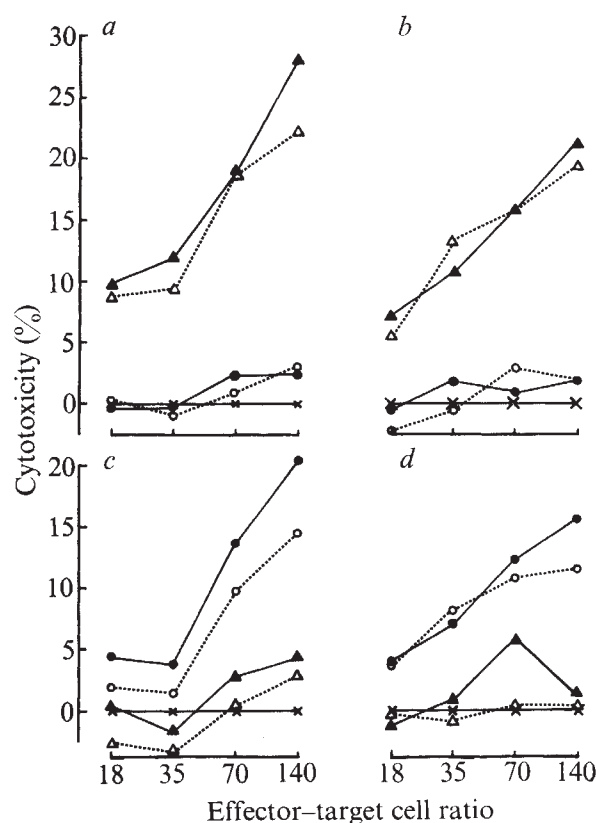
\* Activated with LPS (three experiments) or PHA (five). The results have never indicated a difference between the two kinds of targets, and they have been pooled.

† Cytotoxicity (%) with 140 (one experiment), 80 (one), or 70 (six) effector cells per target cell. Where several ratios were tested in one experiment, 70:1 was used in this analysis.

‡ Wilcoxon's signed rank test (one-tailed).

§ Four experiments with each strain gave the same results and have been pooled.

|| Autologous controls. Seven different donors were used.



**Fig. 1** Human effector cells cause specific  $^{51}\text{Cr}$ -release from mouse target cells carrying the same H-2 SD antigens as the sensitising strain. Targets: *a*, 4R; *b*, 2R; *c*, AQR; *d*, 6R. Human lymphocytes ( $12 \times 10^6$ ) from donor H, purified by Ficoll-Hypaque flotation<sup>10</sup>, were cultured with mitomycin C-treated<sup>11</sup> stimulating cells,  $24 \times 10^6$  autologous lymphocytes,  $\text{H}_m$ , or  $35 \times 10^6$  mouse spleen cells, in upright Falcon No. 3013 flasks in RPMI 1640 with 7.5% heat-inactivated foetal calf serum, penicillin, and streptomycin. After culture for 6 d at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in humidified air and change of 25% of the medium on days 2 and 4, the viable cells were recovered and mixed at the indicated ratios with  $10^4$   $^{51}\text{Cr}$ -labelled target cells and incubated for 3 h according to Alter *et al.*<sup>4</sup>. The target cells were mouse spleen cells stimulated 2 d previously with LPS

the remaining eight are summarised in Table 1. Two features in particular should be noted. First, human lymphocytes sensitised against the I and S regions of the target do not give significantly higher cytotoxicity than lymphocytes sensitised to a different H-2 haplotype (lines 3 and 4, lines 8 and 9). Second, equal killing is obtained when the effector cells are sensitised to the whole H-2 complex or only to the K and D regions of the target (lines 1 and 2, lines 6 and 7). We have obtained similar results on target cells stimulated with LPS (presumably B cells<sup>13</sup>) and PHA (presumably T cells<sup>13</sup>), suggesting that the failure to detect cytotoxicity directed against products of the I and S regions is not an artefact resulting from the tissue distribution of these antigens. Table 1 also shows that there is very little difference between the killing by effector cells sensitised to the non-H-2 antigens of the target and by non-sensitised, cultured cells (lines 4 and 5, lines 9 and 10). If species-specific antigens exist in the man-mouse combination, they must, therefore, play a very minor role for cytotoxicity.

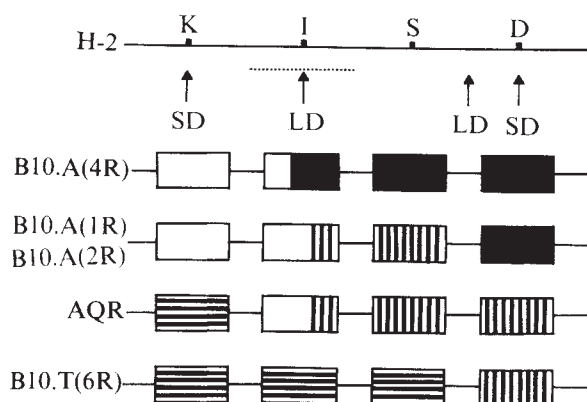
In conclusion, we have shown that of all the antigens expressed on a mouse lymphocyte surface, human effector cells primarily recognise products of the H-2 K and D regions, presumably the SD antigens themselves, which only constitute a few per cent of all surface proteins<sup>14</sup>.

The narrow specificity of cytotoxic effector cells for SD antigens in an allogeneic combination does not seem, therefore, to be the result of a limitation in the number of antigenic determinants which can be recognised, since it is possible to get good cytotoxicity to any number of new determinants presented by xenogeneic cells or generated by chemically modifying syngeneic cells<sup>15</sup>. But in both situations it is primarily the products of the H-2 K and D regions which function as targets. This suggests that there are important biological

(ref. 12); the erythrocytes were removed by hypotonic shock before culturing. Maximum  $^{51}\text{Cr}$ -release was measured after addition of a detergent, control release after addition of unsensitised cultured cells ( $\text{H} + \text{H}_m$ ). The control release ranged from 19.6% to 25.1% of the maximum release. The percentage cytotoxicity was calculated from the amount of  $^{51}\text{Cr}$  released into the supernatant according to the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental release} - \text{control release}}{\text{Maximum release} - \text{control release}} \times 100$$

△, H + 4R<sub>m</sub>; ▲, H + 2R<sub>m</sub>; ●, H + AQR<sub>m</sub>; ○, H + 6R<sub>m</sub>; ×, H + H<sub>m</sub>.



**Fig. 2** H-2 haplotypes of the strains used in this study. 1R and 2R are identical for all the marker loci of H-2 and probably only differ for a chromosomal segment between S and D. They have therefore been used equally in these experiments. 4R, 1R/2R, and 6R are congenic resistant partners carrying different H-2 haplotypes on the same B10 background. AQR was backcrossed four times to C57BL/10 before it was established as an inbred line; it therefore carries a considerable portion of the B10 genome, and studies have shown that it is the H-2 complex of AQR which is most important in allograft reactions with B10 congenic strains<sup>1</sup>. All the strains are as a first approximation considered identical except for their H-2 complexes. The pairs 4R-1R/2R and AQR-6R are SD-identical, but differ in the I and S regions, whereas 1R/2R-AQR are SD-different but identical for the I and S regions.

requirements for target antigens, such as their chemical nature, their density on the cell surface<sup>16</sup> or rate of turnover, which are best fulfilled by the SD antigens. These findings raise the question whether tumour-specific antigens can be recognised by cytotoxic effector cells unless they are associated with or modify the SD antigens of the major histocompatibility complex.

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- <sup>1</sup> Bach, F. H., Widmer, M. B., Bach, M. L., and Klein, J., *J. exp. Med.*, **136**, 1430-1444 (1972).
- <sup>2</sup> Festenstein, H., *Transplant. Rev.*, **15**, 62-88 (1973).
- <sup>3</sup> Schendel, D. J., and Bach, F. H., *J. exp. Med.*, **140**, 1534-1546 (1974).
- <sup>4</sup> Alter, B. J., et al., *J. exp. Med.*, **137**, 1303-1309 (1973).
- <sup>5</sup> Nabholz, M., et al., *Eur. J. Immun.*, **4**, 378-387 (1974).
- <sup>6</sup> Eijssvoogel, V. P., et al., *Transplant. Proc.*, **5**, 1301-1307 (1973).
- <sup>7</sup> Ginsburg, H., *Immunology*, **14**, 621-635 (1968).
- <sup>8</sup> Berke, G., Clark, W. R., and Feldman, M., *Transplantation*, **12**, 237-248 (1971).
- <sup>9</sup> Lindahl, K. F., and Bach, F. H., *Scand. J. Immun.*, **3**, 876 (1974).
- <sup>10</sup> Boyum, A., *Scand. J. clin. Lab. Invest.*, **21**, (suppl. 97), 77-89 (1968).
- <sup>11</sup> Bach, F. H., and Vovnow, N. K., *Science*, **153**, 545-547 (1966).
- <sup>12</sup> Ruhl, H., Vogt, W., Ruhl, Y., Bochart, G., and Schmidt, S., *Immunology*, **25**, 753-760 (1973).
- <sup>13</sup> Greaves, M., and Janossy, G., *Transplant. Rev.*, **11**, 87-130 (1972).
- <sup>14</sup> Vitetta, E. S., Uhr, J. W., and Boyse, E. A., *Eur. J. Immun.*, **4**, 276-282 (1974).
- <sup>15</sup> Shearer, G. M., *Eur. J. Immun.*, **4**, 527-533 (1974).
- <sup>16</sup> Lesley, J., Hyman, R., and Dennert, G., *J. natn. Cancer Inst.*, **53**, 1759-1765 (1974).

## Properdin factor B and histocompatibility loci linked in the rhesus monkey

In man, mouse, monkey and dog the major genetic loci for serologically defined (SD) and mixed lymphocyte reacting (MLR) histocompatibility determinants comprise a closely linked gene complex—the major histocompatibility complex (MHC)—on a single chromosome<sup>1-4</sup>. Genes controlling immune response in mice are located within the MHC<sup>3</sup> and preliminary data for the rhesus monkey suggest that this species also has these genes within or adjacent to the MHC<sup>5,6</sup>. The preservation of the gross structure of this chromosome

region during a long period of mammalian evolution is of considerable biological interest. In addition, the demonstration in the mouse of genetic linkage between MHC and resistance to virus infection<sup>7</sup> and leukaemogenesis<sup>8</sup> and the recognition of association between certain HL-A haplotypes and susceptibility to disease in the human indicates that the MHC may be an area of potentially great practical significance in addition to its role in transplantation.

Genetic polymorphism of properdin factor B<sup>9</sup> is linked to HL-A in man<sup>10</sup>, and the recognition of factor B polymorphism in the rhesus monkey<sup>11</sup> provided the opportunity to test for such linkage in this species. Our results suggest close linkage between the *Bf* (factor B) locus and various genetic systems known to be located in the MHC or RhL-A complex of rhesus monkeys<sup>12,13</sup>.

Animals studied were part of the breeding colony of the Primate Center TNO, Rijswijk. Sera from 76 members of 22 full sibships, which had been sired by one of four males were investigated. RhL-A(SD) haplotypes (there are two closely linked SD loci) were determined on the 22 mothers and four fathers (who were unselected and, as far as can be determined, unrelated) and the 76 progeny as previously described<sup>14</sup>. *Bf* phenotypes were determined<sup>11</sup> without knowledge of SD haplotypes in the 76 progeny, three male and 20 female parents. Two common *Bf* types (*Bf* F and *Bf* S) and four rare types (*Bf* G1, *Bf* G2, *Bf* S1 and *Bf* S2) have been recognised<sup>14</sup> and all except *Bf* G2 and *Bf* S2 were encountered in these pedigrees. Three of the six matings of male No. 381 (*Bf* SS) were uninformative: two with a homozygous female and one with a female of unknown type which produced three homozygous progeny. Data from the 22 sibships examined are presented in Table 1. Nineteen informative matings include those where maternal or paternal *Bf* types were deducible from data of multiple offspring. In these matings at least one parent was heterozygous for *Bf* genes and the data showed that where a particular *Bf* gene could be followed from parent to offspring it was accompanied by the same RhL-A(SD) haplotype. The preliminary assumption made from this observation is that the *Bf* and SD loci lie close together on the same chromosome, that is they are linked. If this is the case, chromosomal crossing over between *Bf* and SD loci during meiosis would disrupt this pattern of linkage (if an odd number of crossovers occurred), producing recombinants. There were only two certain recombinants, both involving maternal chromosomes. In the mating male No. 598 × female No. 832, the gene *Bf*<sup>S</sup> of the female was accompanied by the SD d haplotype in AD and BM and its absence with the c haplotype in CN. In DS it appeared with the c, however, indicating that DS represented a maternal recombinant. In the mating male No. 599 × female No. 493, AX was a maternal recombinant.

Lod (logarithm of the odds) scores were calculated in informative families by standard techniques<sup>15</sup>. The lod score in males was 10.1 for a recombination fraction ( $\theta$ ) of zero and in females had a maximum value of 5.6 ( $\theta = 0.04$ ) so that the probability of linkage is very high. In the total population the maximum lod score was 15.1 ( $\theta = 0.02$ ) and the odds in favour of linkage as opposed to a chance association exceed  $10^{13}$  to 1. Corrections for prior probabilities have not been included. Three of the four males were heterozygotes for *Bf* and the most likely phase (whether, in a given animal, specific SD and *Bf* genes are on the same or different chromosomes) can therefore be deduced in one mating. Lod scores in males would be even higher if this information were taken into account. So far no paternal recombinants have been detected but a higher incidence in females is frequently found in linkage analysis<sup>16</sup>. Since the most likely recombination fraction in the pooled data is 0.02, this, if correct, would indicate a map distance of 2 centimorgans between *Bf* and the region coding for serologically defined antigens of RhL-A. No evidence for linkage disequilibrium has been found in these studies.

In each of two matings with male No. 599, one paternal recombination between first and second SD series of RhL-A



**Table 1** Genotyping for serologically defined (SD) antigens of RhL-A and properdin factor B (Bf) genes in rhesus monkey families

| Parents and offspring | SD* haplotype | Bf type | Parents and offspring | SD haplotype | Bf type | Parents and offspring | SD haplotype | Bf type | Parents and offspring | SD haplotype | Bf type |
|-----------------------|---------------|---------|-----------------------|--------------|---------|-----------------------|--------------|---------|-----------------------|--------------|---------|
| ♂381                  | ab            | SS      | ♂598†                 | ab           | (S1F)   | ♂599                  | ab           | FS      | ♂600                  | ab           | SF      |
| ♀584                  | cd            | SS      | ♀834                  | cd           | FS      | ♀581                  | cd           | FF      | ♀669                  | cd           | SS      |
| V                     | bd            | SS      | UU                    | bc           | FF      | G                     | bc           | SF      | AC                    | ad           | SS      |
| JJ                    | bc            | SS      | BD                    | bc           | FF      | GG                    | bc           | SF      | BI                    | ad           | SS      |
| AV                    | bc            | SS      | CS                    | ad           | S1S     | AG                    | ac           | FF      | CR                    | ad           | SS      |
| BU                    | bc            | SS      | EK                    | bc           | FF      | CF                    | ac           | FF      | EL                    | bc           | FS      |
| CZ                    | bc            | SS      |                       |              |         | DV                    | bc           | SF      | FS                    | bc           | FS      |
| ♀432                  | cd            | G1F     | ♀730                  | cd           | SG1     | ♀1115                 | cd           | SS1     | ♀603                  | cd           | FS      |
| N                     | ac            | SG1     | FF                    | bd           | FG1     | CL                    | a/bc         | FS      | T                     | bd           | FS      |
| AK                    | bd            | SF      | BT                    | ac           | S1S     | EF                    | bd           | SS1     | DF                    | bd           | FS      |
| CP                    | ad            | SF      | DA                    | ad           | S1G1    | GZ                    | ac           | FS      | FO                    | bd           | FS      |
| FN                    | ac            | SG1     |                       |              |         |                       |              |         | GY                    | bc           | FF      |
| ♀852                  | cd            | FS      | ♀832                  | cd           | FS      | ♀498                  | cd           | SF      | ♀597                  | cd           | FF      |
| AH                    | ac            | SF      | AD                    | bd           | FS      | NN                    | ac           | FS      | AB                    | bc           | FF      |
| BL                    | bd            | SS      | BM                    | ad           | S1S     | AQ                    | ac           | FS      | BF                    | bc           | FF      |
| DN                    | ac            | SF      | CN                    | ac           | S1F     | ED                    | a/bd         | FF      | CG                    | ad           | SF      |
| EV                    | ad            | SS      | DS                    | bc           | FS←     |                       |              |         | DW                    | ac           | SF      |
| ♀594†                 | cd            | (.S)    | ♀589†                 | cd           | (SS1)   | ♀590                  | cd           | SS      | ♀429                  | cd           | G1F     |
| YY                    | ad            | SS      | Z                     | ac           | S1S     | CV                    | bd           | SS      | XX                    | bc           | FG1     |
| BJ                    | bd            | SS      | AE                    | ad           | S1S1    | EI                    | ad           | FS      | BG                    | ac           | SG1     |
| FU                    | bd            | SS      | BP                    | bc           | FS      | GD                    | bd           | SS      |                       |              |         |
|                       |               |         |                       |              |         | HG                    | ac           | FS      |                       |              |         |
| ♀494                  | cd            | SF      | ♀728                  | cd           | G1S     | ♀493                  | cd           | G1F     |                       |              |         |
| L                     | ac            | SS      | KK                    | ad           | S1S     | AX                    | bc           | SF←     |                       |              |         |
| AA                    | bd            | SF      | AS                    | bd           | FS      | CK                    | bc           | SG1     |                       |              |         |
| AM                    | ad            | SF      | EP                    | ac           | S1G1    | DM                    | bc           | SG1     |                       |              |         |
| CY                    | ac            | SS      |                       |              |         |                       |              |         |                       |              |         |
| ♀324                  | cd            | S1S1    | ♀306                  | cd           | FS      | ♀926                  | cd           | SS      |                       |              |         |
| CU                    | bc            | SS1     | SS                    | bc           | FF      | DC                    | bc           | SS      |                       |              |         |
| EM                    | ac            | SS1     | BK                    | ac           | S1F     | FJ                    | ac           | FS      |                       |              |         |

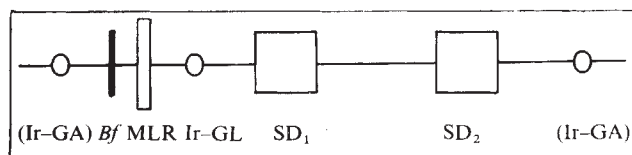
\*By convention, a and b are paternal, c and d maternal haplotypes; for details of SD haplotyping, see ref. 12.

†Animal dead.

( ) Bf type 'deduced' when possible. Arrows indicate suggested recombinants, see text.

was encountered<sup>12</sup>. In each case, the Bf type accompanied the first segregant series SD type, suggesting that the Bf locus lies on the first series side of the complex. Unfortunately, matings in which recombination occurred between the SD region and the major MLR locus of RhL-A were uninformative for Bf linkage. MLR, however, probably also maps on the side of the first RhL-A(SD) locus<sup>17</sup> and if this is confirmed, the following reasoning can be applied. AX is a maternal recombinant (Table 1), being of different Bf type but SD identical to her siblings CK and DM. In MLR, AX also fails to stimulate or be stimulated by her siblings, indicating lack of recombination between SD and the major MLR loci<sup>17</sup>. DS has also been shown not to be a recombinant between the SD and major MLR loci. These observations can best be explained by placing the MLR locus between Bf and SD or by placing SD between Bf and MLR. Since both Bf and MLR seem to be on the first segregant series side of the rhesus SD complex, the former situation (Fig. 1) is the more likely. Preliminary data in humans (Ch. Rittner, personal communication) indicate that MLR is between SD and Bf in this species.

**Fig. 1** Possible map of the chromosomal region containing the major histocompatibility or RhL-A complex in the rhesus monkey. Ir-GA and Ir-GL, immune response genes. Bf, properdin factor B structural locus. SD, two loci for serologically defined histocompatibility antigens. MLR, major locus for mixed lymphocyte reactivity. Evidence placing MLR nearer to first segregant SD series (SD<sub>1</sub>) remains tentative. Note that Ir-GA has been placed on the outside of the complex but further localisation is not possible.



In the mating male No. 598 × female No. 834, EK has been found to be a recombinant between SD and Ir-GA (a gene controlling immune responsiveness to the linear synthetic polymer of *L*-glutamic acid and *L*-alanine)<sup>6,18</sup>. Since EK does not show recombination between Bf and RhL-A(SD), it seems that either the Bf locus lies between SD and Ir-GA or the SD region lies between Bf and Ir-GA. A possible situation, suggested by available data, is summarised by Fig. 1.

The HL-A complex of humans lies on the sixth autosome<sup>19,20</sup> and includes the LA and FOUR series (SD) which are closely linked to MLR, Bf, as well as to PGM<sub>3</sub><sup>16</sup>, ME1 and IPO-B<sup>19</sup>. There may also be a human Ir (immune response) region in the MHC. A region showing striking homology to the human MHC exists in the rhesus monkey, and appears to have been conserved through much of mammalian evolution. Bf, apparently closely associated with the MHC, will provide a convenient reference marker for other linkages.

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- Balner, H., Gabb, B. W., Dersjant, H., van Vreeswijk, W., and van Rood, J. J., *Nature*, **230**, 177 (1971).
- Bach, J. F., Widmer, M. B., Bach, M. L., and Klein, J., *J. exp. Med.*, **136**, 1430 (1972).
- Klein, J., Widmer, M. B., Segall, M., and Bach, H. F., *Cell Immun.*, **4**, 442 (1972).
- Vriesendorp, H. M., D'Amato, J., Epstein, R. B., Westbroek, D. L., and van Rood, J. J., *Transplant. Proc.*, **5**, 311 (1973).

- <sup>5</sup> Balner, H., Dorf, M. E., de Groot, M. L., and Benacerraf, B., *Transplant. Proc.*, **5**, 1555 (1973).
- <sup>6</sup> Dorf, M. E., Balner, H., de Groot, M. L., and Benacerraf, B., *Transplant. Proc.*, **6**, 119 (1974).
- <sup>7</sup> Oldstone, M. B. A., Dixon, F. J., Mitchell, G. F., and McDevitt, H. O., *J. exp. Med.*, **137**, 1201 (1973).
- <sup>8</sup> Lilly, F., *Natn. Cancer Inst. Monogr.*, **22**, 631 (1966).
- <sup>9</sup> Alper, C. A., Boenisch, T., and Watson, L., *J. exp. Med.*, **135**, 68 (1972).
- <sup>10</sup> Allen, F. H. Jr., *Vox Sang.*, **27**, 382 (1974).
- <sup>11</sup> Ziegler, J. B., Watson, L., and Alper, C. A., *J. Immun.* (in the press).
- <sup>12</sup> Balner, H., *Transplant. Rev.*, **15**, 50 (1973).
- <sup>13</sup> Balner, H., and van Vreeswijk, W., *Transplant. Proc.* (in the press).
- <sup>14</sup> Balner, H., Gabb, B. W., Toth, E. K., Dersjant, H., and van Vreeswijk, W., *Tissue Antigens*, **3**, 257 (1973).
- <sup>15</sup> Cavalli Sforza, L. L., and Bodmer, W. F., in *The Genetics of Human Populations*, **870** (Freeman, San Francisco, 1971).
- <sup>16</sup> Lamm, L. U., Sveigaard, A., and Kissmeyer-Nielsen, F., *Nature new Biol.*, **231**, 109 (1971).
- <sup>17</sup> Balner, H., and Toth, E. K., *Tissue Antigens*, **3**, 273 (1973).
- <sup>18</sup> Dorf, M. E., Balner, H., and Benacerraf, B., *Transplant. Proc.* (in the press).
- <sup>19</sup> van Someren, H., et al., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 962 (1974).
- <sup>20</sup> Lamm, L. U., et al., *Human Heredity*, **24**, 273 (1974).

## Follicle stimulating hormone enhances attachment of rat testis cells in culture

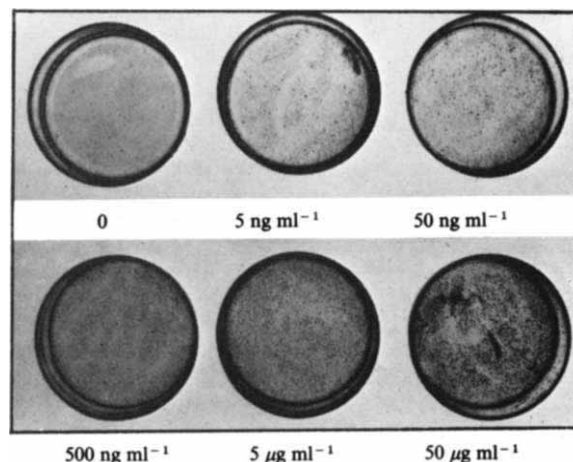
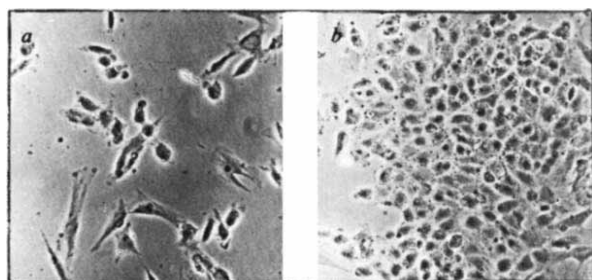
EXTENSIVE *in vivo* experiments indicate that mammalian spermatogenesis is under the regulation of pituitary gonadotrophins. The cellular sites and mechanisms by which spermatogenesis is regulated by gonadotrophic hormones, however, are poorly understood. Means has shown *in vivo* that follicle stimulating hormone (FSH) stimulates several cellular processes<sup>1,2</sup>. So far, however, no one has shown an *in vitro* effect of FSH on the testis. Several reports have indicated that hormones or hormone mediators alter cell properties in culture. In particular, testosterone was found to cause morphological changes in CHO cells<sup>3</sup> and cyclic adenosine 3',5'-monophosphate has been shown to affect the adhesive properties of L-929 (ref. 4) and BHK (ref. 5) cells. We have been studying the growth of testis cells from the 10-d-old rat in culture and report here our observations on the stimulation of testicular cell attachment to culture dishes by follicle stimulating hormone.

Cells from 14 testes were dissociated by continuous agitation in 0.1% trypsin (Gibco, 1:250) for 50 min in a trypsinising flask. The tissue-free supernatant was collected at 10-min intervals and diluted with 8% foetal calf serum (1:1). Cells were collected by centrifugation at 1,000g for 10 min and suspended in NCTC-135 media (Gibco) containing 10% foetal calf serum.

Isolated testis cells ( $5 \times 10^5$  per dish) were incubated for 24 h in 35-mm dishes (Falcon) in 2 ml of NCTC-135 with foetal calf serum, (10%), penicillin ( $100 \text{ U ml}^{-1}$ ), and streptomycin ( $100 \mu\text{g ml}^{-1}$ ) at  $31.5^\circ\text{C}$  in an air- $\text{CO}_2$  atmosphere (95:5). Culture dishes contained either FSH (NIH-FSH-S<sub>8</sub>), human chorionic gonadotrophin (HCG, Ayerst  $531 \text{ U mg}^{-1}$ ), or bovine serum albumin (BSA). In dishes without FSH, individual fibroblast-like cells were seen on the bottom of the dish (Fig. 1a) whereas in the dishes containing FSH, colonies containing round, tightly-packed cells were seen (Fig. 1b).

After incubation, the dishes were washed twice with 2 ml of phosphate buffered saline to remove unattached cells, fixed for 12 h in 10% formalin, and stained for 5 min in haema-

**Fig. 1** a, Phase contrast photomicrograph of cells growing in culture without FSH. b, Phase contrast photomicrograph of a cell colony growing in culture with FSH ( $\times 160$ ).



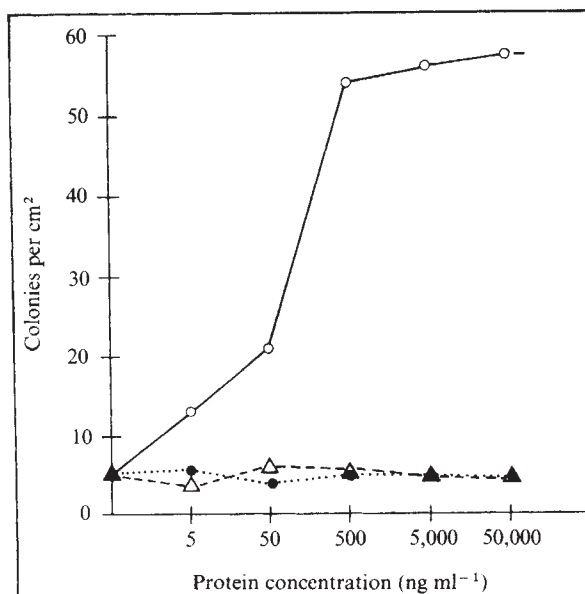
**Fig. 2** Photograph of haematoxylin-stained colonies in dishes treated with various concentrations of FSH after 24 h in culture.

toxylin. Examination of stained colonies on the bottom of the dish indicated a dose-dependent ( $0$ – $50 \mu\text{g ml}^{-1}$ ) stimulation of colony number by FSH (Fig. 2).

To examine more closely the effect of FSH on colony formation, cultures were treated with FSH, HCG or BSA at  $5$ – $50,000 \text{ ng ml}^{-1}$ ; triplicate points were run at each dose in three experiments. Stained dishes were then placed on a  $4 \times 4$ -cm grid and the colonies in  $5\text{-cm}^2$  areas chosen from a random number table were counted at  $\times 6$  magnification under a dissecting microscope. Colony counts are summarised in Fig. 3, and show a clear dose-dependent effect of FSH ( $P < 0.01$ , analysis of variance) on colony number while HCG and BSA had no significant effect. Furthermore, in subsequent experiments, HCG had no effect on colony number at doses up to  $5 \text{ mg ml}^{-1}$  ( $2,655 \text{ IU}$ ).

In the same experiment the effect of the various treatments on the size (number of cells) of the colonies was determined.

**Fig. 3** The effect of FSH ( $\circ$ ), HCG ( $\bullet$ ) and BSA ( $\Delta$ ) on colony density after 24 h in culture. Dishes were stained with haematoxylin and the colonies in  $5\text{-cm}^2$  chosen at random on the bottom of the dish were counted. The average of the five determinations for three dishes represented one experimental value. The points are an average of the three experiments.





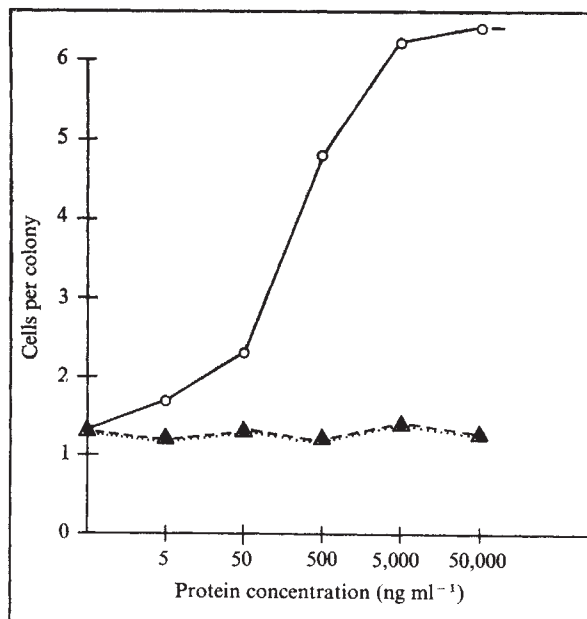


Fig. 4 The effect of FSH (○), HCG (●) and BSA (△) on colony size after 24 h in culture. Dishes were stained with haematoxylin and cells per 100 colonies were counted at  $\times 200$ . The average number of cells per colony for three dishes represented one experimental value. The points are the average of three experiments. Note: △ and ● are superimposed.

Cells were counted at  $\times 200$  magnification on 100 colonies on the bottom of the dish. As with colony density, colony size increased with FSH but not with BSA or HCG (Fig. 4). As before, the effect of FSH was dose-dependent and analysis of variance indicated a highly significant treatment effect ( $P < 0.01$ ).

The results indicate that FSH has a stimulatory effect on some testicular cell in culture. Our preparation of testes cells from the 10-d-old rat contained more than 60% preleptotene spermatocytes; however, these cells do not attach to culture dishes<sup>6</sup> and therefore do not seem to have been the cells stimulated with FSH. Extensive electron microscopic examination of the cells in colonies indicated that they were not germinal cells and were a single cell type; however, an exact identification of the cell type involved would be premature at this time.

Our observation of an action of FSH on a testicular cell *in vitro* agrees with the observations that FSH (1) stimulates RNA synthesis<sup>1</sup> and protein kinase activity<sup>2</sup> and (2) is involved in the initiation of spermatogenesis<sup>7</sup> in the rat testis *in vivo*. The observation that HCG does not stimulate colony formation suggests that the *in vitro* effect may be, like the *in vivo* effect, hormone specific. These findings demonstrate an action of FSH on a discrete testicular cell *in vitro* and should provide new insight into hormonal and cellular interactions involved in spermatogenesis.

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<sup>1</sup> Means, A., *Endocrinology*, **89**, 981 (1971).

<sup>2</sup> Means, A. R., MacDougall, E., Soderling, T., and Corbin, J., *J. biol. Chem.*, **249**, 1231 (1974).

<sup>3</sup> Hsie, A., Puck, T., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 358 (1971).

<sup>4</sup> Funabiki, R., and Cassens, R. G., *Nature*, **236**, 247 (1972).

<sup>5</sup> Grinnell, F., Millens, M., and Steve, P. A., *Nature*, **241**, 82 (1973).

<sup>6</sup> Steinberger, A., *Anat. Rec.*, **151**, 420 (1965).

<sup>7</sup> Lostroh, A. J., *Acta Endocrinol.*, **43**, 552 (1963).

## Ultrastructure of muscle spindles in dystrophic mice

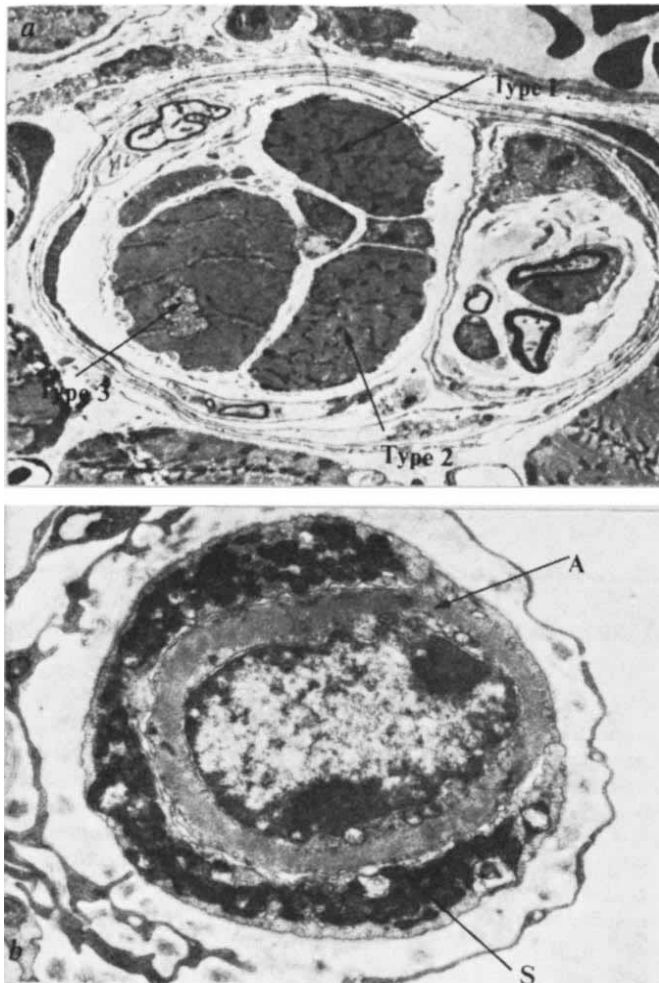
ALTHOUGH the pathological changes in muscular dystrophy are commonly attributed to a primary abnormality of the muscle fibres, there is evidence to suggest that muscular dystrophy may be attributable to a neuronal abnormality<sup>1</sup>. This has become known as the 'neural hypothesis'. Dystrophic mice (Bar Harbor 129 REJ *dy/dy*) provide a useful model of muscular dystrophy for experimental study. In spite of the early onset of abnormal reflexes in muscular dystrophy, the richly innervated sensory organs, muscle spindles, have received relatively little attention<sup>2,3</sup>. Optical microscopical studies have indicated that spindles in dystrophic mouse muscles are essentially unaffected<sup>4</sup>, although in some human diseases they seem to undergo minor changes<sup>5</sup>. This study was undertaken to establish whether there are ultrastructural changes in spindles of dystrophic mice which are detectable only by electron microscopy.

In dystrophic muscle each spindle was found to possess a prominent periaxial space containing a characteristic flocculent precipitate. The periaxial space was bounded by a capsule containing up to six layers of perineurial epithelial cells. Each perineurial epithelial cell contained numerous pinocytotic vesicles. The capsule enclosed the muscle fibres of the spindle for most of their lengths except close to their insertions into perimysium. Capillaries were never seen to penetrate the capsule and enter the periaxial space.

The dystrophic spindles contained an average of three to eight intrafusal fibres. No spindle contained less than three intrafusal fibres. Nuclear bag fibres contained up to three nuclei and a peripheral rim of myofilaments in each equatorial cross section and could be clearly differentiated from the narrower and shorter nuclear chain fibres, which contained singly occurring central nuclei. Both types of intrafusal fibre contained numerous myofilaments in their polar regions. Nuclear bag fibres tended to contain confluent masses of myofilaments interspaced with occasionally occurring mitochondria of variable size. In a few nuclear bag fibres the mitochondria were of more regular size and more evenly dispersed throughout the fibre cross section. These two types of intrafusal fibre correspond to those designated types 2 and 3 in other species<sup>7</sup>. Nuclear chain fibres contained groups of myofilaments which formed discrete myofibrils often separated by numerous mitochondria. In both nuclear bag and nuclear chain fibres the number of myofilaments was markedly reduced in equatorial compared with polar regions. Dilated terminal cisternae of the triads typical of those found in other species<sup>8</sup> were also found.

Satellite cells were found lying between the basement membrane of the intrafusal fibres and their plasma membranes. They seemed inactive because their relatively small amounts of cytoplasm contained few ribosomes and a little rough endoplasmic reticulum. All the intrafusal fibres examined received a direct sensory innervation without intervening basement membrane. Two motor-nerve endings were also observed. Their primary synaptic clefts contained a continuous layer of basement membrane which extended into the relatively few secondary synaptic clefts that occurred.

None of the spindles in dystrophic muscles was seen to contain any ultrastructural abnormality and did not differ significantly from the spindles in the control muscles. These findings have important implications for the 'neurogenic hypothesis' of muscular dystrophy. If that hypothesis is true then changes in the richly innervated intrafusal fibres may be expected. Consequently, at least a modification of the hypothesis must be proposed. Several possibilities exist; for example, either there is a selective sparing of the sensory or  $\gamma$ -motor supply of



**Fig. 1** *a*, Transverse section across the polar region of a muscle spindle in a clinically dystrophic mouse. Three types of intrafusal fibre can be differentiated. Type 1 fibres (nuclear chain) contain many large mitochondria; type 2 fibres (nuclear bag) contain intermediate numbers of evenly scattered mitochondria; type 3 fibres (nuclear bag) contain a few small mitochondria. ( $\times 2,300$ ). *b*, Transverse section across the equatorial region of the nuclear chain (type 1) fibre seen in (*a*). A primary sensory ending (S) contains numerous mitochondria and partly encircles the intrafusal fibre. The cross sectional area (A) which contains myofibrils in this region of the fibre is much smaller than in the polar regions. ( $\times 12,000$ ). Specimens of soleus and extensor digitorum longus muscles were obtained from six clinically affected Bar Harbor 129 REJ *dy/dy* dystrophic mice and from six non-littermate controls, and prepared for electron microscopy as described previously<sup>6</sup>. Eleven spindles were found in dystrophic muscle and eight in control muscle. Ultrathin sections were cut from these spindles at intervals of 25–50  $\mu\text{m}$  along most of their lengths. A few spindles were also examined in longitudinally cut sections.

the spindles or, alternatively, the intrafusal fibres may have acquired a high resistance to the supposed neurogenic influence. In support of the latter, in many human muscle diseases, apart from an apparent disuse atrophy, the intrafusal muscle fibres usually seem to be relatively unaffected<sup>9,10</sup>. Although the relevance of such findings in dystrophic mice to the pathogenesis of human muscular dystrophy is uncertain, studies on human dystrophic spindles could be of considerable interest.

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<sup>1</sup> McComas, A. N., *Nature*, **251**, 569–570 (1974).

- <sup>2</sup> Meier, H., *Experientia*, **25**, 965 (1969).
- <sup>3</sup> Harris, J. B., Wallace, C., and Wing, J., *J. neurol. Sci.*, **15**, 245–249 (1972).
- <sup>4</sup> Yellin, H., *Experientia*, **30**, 286–287 (1974).
- <sup>5</sup> Swash, M., and Fox, K. P., *J. neurol. Sci.*, **22**, 1–24 (1974).
- <sup>6</sup> James, N. T., and Meek, G. A., *J. Ultrastruct. Res.*, **43**, 193–204 (1973).
- <sup>7</sup> James, N. T., *Histochem. J.*, **3**, 456–462 (1971).
- <sup>8</sup> James, N. T., and Meek, G. A., *J. Anat.*, **116**, 219–226 (1973).
- <sup>9</sup> Cuzzato, G., and Walton, J., *J. neurol. Sci.*, **7**, 15–70 (1968).
- <sup>10</sup> Patel, A., Lalitha, V., and Dastur, D., *Brain*, **91**, 737–750 (1968).

## Squint and the development of binocularity in humans

IN the past ten years it has been shown that binocularity<sup>1</sup>, orientation sensitivity<sup>2,3</sup>, ocular dominance<sup>4</sup> and disparity<sup>5</sup> of cortical neurones can be modified by experimental procedures in kittens during the sensitive period (3–14 weeks of age).

In 4-week-old kittens which had one eye closed for 6 d the percentage of binocular cells decreased to 9% from 80–90% in normal controls, but closing one eye for 2 months in 4-month-old kittens did not affect the relative number of binocular and monocular cells<sup>6</sup>. In humans, according to clinical experience, binocular vision is not fully established at birth and develops to full maturity during the first four to five years of life<sup>7</sup>.

If neurones of the visual cortex need to be excited by identical or nearly identical stimuli from both eyes during the critical period in order to develop binocularity, a squint during this period should result in a smaller number of cells than normal with binocular input. Since binocular neurones of the visual cortex are an essential prerequisite for binocular single vision and depth perception<sup>8</sup>, a lack of such cells will result in a failure of real binocular single vision after correction of the squint. This is also supported by clinical observations which indicate that the later a squint is acquired the more likely it is that binocular vision will be restored after a successful operation<sup>9</sup>.

We have tried to correlate these clinical observations with quantitative measurements of interocular transfer in children whose squint had developed at different ages but was corrected by surgery. This was done with the assumption that interocular transfer may be a reliable indicator for the binocular input to visual cortical neurones. A simple test of this kind is the interocular transfer of the tilt after-effect described by Gibson<sup>10</sup>. If a subject fixates a grating tilted 10° anticlockwise away from the vertical for some minutes, he will make a systematic clockwise error when asked to adjust the same grating in the vertical position. The same systematic error is found, though to a smaller degree, if one eye is trained and then adjustment of the grating is done with the other, untrained eye (binocular transfer). Patients with strabismus have a smaller after-effect transferred from one eye to the other than subjects with normal vision<sup>11</sup>.

Twelve children, 5.10 to 15.2 years old, served as subjects. Nine of them (mean age 9.8 yr) had been patients of the Göttingen eye hospital and had suffered from strabismus concomitans convergens alternans in early life. They had been operated on at the age of 3–5 yr (mean 4.1 yr). Preoperation squinting angle was between 12° and 40° (mean 23°). Binocular interaction was tested on the synoptophor, visual acuity with the Pflüger-hook. The Worth test and the Maddox cross test were also applied. The Gibson transfer phenomenon was then tested on the synoptophor. Three children with normal vision (mean age 5.9 yr) served as control subjects.

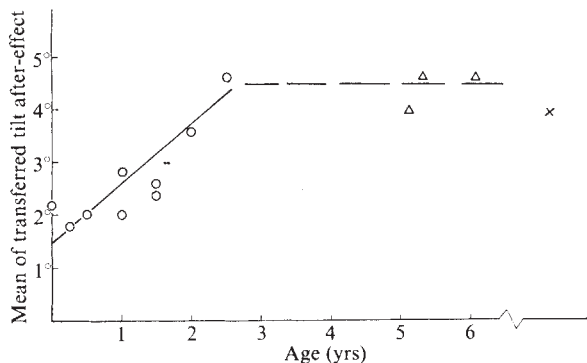
A vertical light bar on a black ground was adjusted five times for each eye and the angular error measured. This was of the order of  $\pm 0.5^\circ$ . The subject then adapted monocularly for 2.5 min to a grating orientated 10° anticlockwise to the vertical. The bar had the same width as the lines of the grating. Immediately after this adaptation period the single bar, set at 5° clockwise to the vertical was shown to the untrained eye. The experimenter slowly rotated the single line and asked the subject to say 'stop' when the line appeared vertical to him. Normal children indi-



cated that the bar appeared vertical when it was still tilted about 4–5° from the normal. This value will be called transferred tilt after-effect (TTA). After readaptation for 45 s the test was repeated twice for each eye. The typical error between successive measurements was less than  $\pm 0.3^\circ$ .

The age at which the squint first appeared was noted in the hospital records and the parents were also asked. Figure 1 shows that the TTA is smallest (1–2°) in children who had a squint from birth. It increases steadily the later the squint started. A child who had started squinting at the age of 2.6 yr showed no difference in TTA compared with subjects with normal vision. The TTA values shown on the ordinate of Fig. 1 are the means of the TTA values from the right to the left and from the left to the right eye.

Only 3 children were used as controls. Their TTA values were the same as those known for normal subjects in other investigations<sup>12</sup>, so that further controls were not necessary. Ophthalmological assessment of binocularity closely corresponded to these results. The children who had started squinting at birth had simultaneous perception only (stage 1 of binocularity). Those who had acquired their squint at 0.6–1 yr could fuse images (stage 2), and those whose squint began at 1.6 yr or later could perceive depth (stage 3).



**Fig. 1** Binocular transfer of tilt after-effect in operated strabismic children, whose squint developed at different ages. Abscissa: ○, age of onset of squint in patients; △, age of control children at which the measurement was done. Ordinate: mean error of adjustment of vertical line with one eye after exposure of the other eye to a grating tilted 10° away from the vertical for some minutes (transfer of tilt after-effect). ×, Control values from Campbell and Maffei<sup>12</sup>.

Thus the later a squint began in the child the higher was his mean interocular transfer angle, and the better his binocular vision. If the squint started by the age of approximately 2.6 yr, normal interocular transfer was possible. These findings indicate that at an age of about 2.6 yr, binocular functions of the visual cortex as measured with the TTA are well enough established to be restored by correction of a squint. The age of about 2–2.6 yr may therefore be considered the end of the critical period for the development of binocular vision in humans.

The fact that the interocular transfer has never been found to be zero could indicate either that at least some binocular cortical neuronal connections develop independently of binocular vision during early life time, or that a post-operation training effect took place. Orthoptistic training is given to children until they are nine years old.

The good correspondence between the TTA results and the clinical assessment of binocularity suggests that the TTA is a good indicator of binocular functioning of the visual cortex. It may be directly related to the number of binocular units in the visual cortex as also suggested by earlier investigations<sup>11,13</sup>. As it is a simple test it could be included in clinical testing of binocular functions.

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- Hubel, D. H., and Wiesel, T. N., *J. Neurophysiol.*, **28**, 1041–1059 (1965).
- Blakemore, C., and Cooper, G. F., *Nature*, **228**, 477–478 (1970).
- Hirsch, H. V. B., and Spinelli, D. N., *Expl Brain Res.*, **13**, 509–527 (1971).
- Wiesel, T. N., and Hubel, D. H., *J. Neurophysiol.*, **28**, 1029–1040 (1965).
- Shlaer, S., *Science*, **173**, 638–641 (1971).
- Dews, P. B., and Wiesel, T. N., *J. Physiol.*, **206**, 437–455 (1970).
- Engelking, E., *Grundriss der Augenheilkunde*, (Springer, Berlin, 1964).
- Nikara, T., Bishop, P. O., and Pettigrew, J. D., *Expl Brain Res.*, **6**, 353–372 (1968).
- Holland, G., *Schielbehandlung*, **2**, 30–40 (1971).
- Gibson, J. J., *J. exp. Psychol.*, **16**, 1–31 (1933).
- Movshon, J. A., Chambers, B. E. I., and Blakemore, C., *Perception*, **1**, 483–490 (1972).
- Campbell, F. W., and Maffei, L., *Vision Res.*, **11**, 833–840 (1971).
- Mitchell, D. E., Ware, C., *J. Physiol., Lond.*, **236**, 707–721 (1974).

## Visual acuity development coincides with the sensitive period in kittens

At about ten days of age when a kitten's eyes open, synaptic connections of its visual system are partly laid down<sup>1</sup>. They are completely developed during the sensitive period<sup>2</sup> which lasts from about three weeks of age to three months. During this period of plasticity the formation and modification of synaptic connections respond to features in the visual environment<sup>3–6</sup>. Both the original and subsequently developed connections may be disrupted or modified by environmental factors during the sensitive or critical period with a sensitivity that peaks at about the fourth week and gradually drops off<sup>2</sup>.

Conventional (psychophysical) visual acuity as well as its electrophysiological acuity correlate, obtained by pattern-dependent visual evoked potentials, must also develop in the kitten as a result of the organisation of synaptic connections. (Visual evoked potential acuity measurements are based on electrical processing in the visual cortex, although it is possible that they may reflect retinal and geniculate processing resolution before cortical processing takes place.) Our goal was to measure visual acuity in cats and kittens of various ages to determine the time course of its development.

Visual evoked potential acuity measurements were made on nine kittens and three adult cats, all raised in a normal visual environment. The data were obtained from the two eyes separately in each of four kittens and in one eye in the rest of the kittens and cats, providing fifteen data points in all. A horizontal rather than longitudinal time study was dictated because stable electrodes were difficult to maintain through the thin, growing skull of the kittens. Following the method of Rose *et al.*<sup>7</sup>, stainless steel screw electrodes were positioned just through the skull over the visual cortex and, with dental acrylic cement, were encased in a small electric connector on the head. The implantation of electrodes yielded excellent potentials with a much better signal to noise ratio than did surface electrodes.

The responses were evoked by the virtually instantaneous reversal of the light and dark stripes of a bar grating pattern by an optical electromechanical shaker. A constant temporal frequency of 0.5 Hz was used with a variable bar width (spatial frequency). During recordings the animals were quiescent under general anaesthesia (sodium pentobarbital) and a contact lens protected the optical quality of the cornea. It is clear that the optical quality of the eye of the very young kitten is inferior to that of the adult. But on the basis of ophthalmoscopic observation we feel that it is unlikely that optical factors limit the acuity determined for the younger animals. The eye was aligned to the screen by finding the optic disc ophthalmoscopically and projecting it on to the screen. Retinoscopy and trial lenses provided

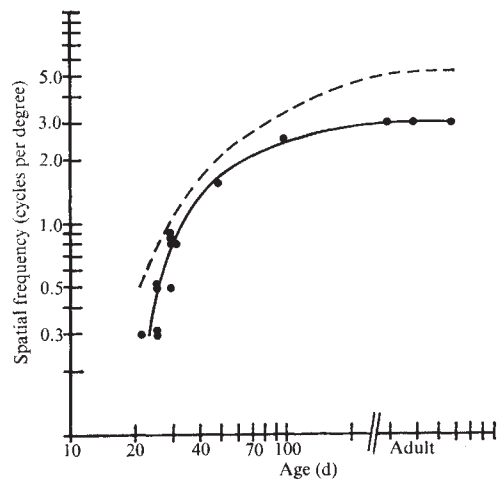
an optical correction to the screen at 57 cm which covered a  $40^\circ$  visual angle. The target had 80% contrast and a luminance of  $24 \text{ cd m}^{-2}$ .

For each size or spatial frequency of grating, 100 responses were recorded and averaged on a PAR Waveform Eductor to provide a good signal to noise ratio (Fig. 1). Measurements were begun at 23 days of age, well after the hyaloid artery disappeared and the optical media were clear. The amplitude of the evoked response was found to be a function of the spatial frequency of the pattern, confirming Berkley and Watkins<sup>8</sup>. Visual acuity was taken as the highest spatial frequency for which a clear electrical response was present. Spatial frequency thresholds for each animal were plotted on a log-log scale as a function of age (Fig. 2, solid curve).

The points on the curve are, in principle, slightly above threshold because a clear though minimum response was used as the criterion. At the next higher step of spatial frequency no response could be observed, so the exact threshold cannot be above these points which are represented by the dashed curve of Fig. 2. This curve is at or slightly below threshold and is the limit of the maximum possible error in the minimum threshold as determined by this method.

Grating resolution of the adult cats fell between 3 and 5 cycles per degree which is similar to that found by Berkley and Watkins<sup>8</sup> and by Campbell *et al.*<sup>9</sup> using related evoked potential methods, and by Muir and Mitchell<sup>10</sup> using a standard psychophysical method. Recently Blake *et al.*<sup>11</sup> using conditioned suppression were able to approach 6 cycles per degree. Wässle<sup>12</sup> found a comparable value for the optical upper spatial frequency cut-off using two parallel slits for the cat's eye, calculated at 4 to 5 arcmin or 6 cycles per degree. Pure optical values would be expected to be the same or higher than the psychophysical acuity value of 3 to 5 cycles per degree. These values seem low when compared with those of the human eye; the cat's optical system, however, is poorer<sup>10</sup> and its retina lacks a fovea centralis.

It is clear from the trend of the curve that from 23 days of age, development of visual acuity in the cat is exceedingly rapid, and is virtually complete by 100 d. At 23 d of age acuity was only 0.3 cycles per degree or 20/2000 Snellen. Ten days later the acuity had improved to 1 cycle per degree or 20/600, an



**Fig. 2** The highest spatial frequency for which an evoked potential was present is graphed as a function of age (closed circles). A curve has been fitted by eye to these points (solid line). The dashed line joins the next higher spatial frequencies for which no pattern evoked potentials were present and is a measure of the maximum error. Age scale for adult cats is in yr.

enormous increase. Between 33 d and 100 d the acuity approached the adult level of 3 cycles per degree, or 20/200.

The optical media may become slightly clearer during the third to sixth week which could make a curve representing synaptic development slightly less steep. Fig. 2 may also be described as follows: It "... begins suddenly near the start of the fourth week, at about the time a kitten begins to use its eyes, and persists until some time between the sixth and eighth weeks; it then begins to decline, disappearing ultimately around the end of the third month." This is Hubel and Wiesel's description<sup>2</sup> of the sensitive period for changes in ocular dominance which fit our data. Their descriptive data have been plotted by Blakemore<sup>13</sup>. While other functions of the visual system may have somewhat different sensitive periods, it seems likely that all other functions fit within the limits of that described above for ocular dominance.

Because the normal development of acuity takes place during the sensitive period, our findings support the hypothesis that this development may be used as a measure of the visual sensitive period. A similar but non-invasive method (that is, using electrodes on the skin rather than on the brain) can be used to investigate the visual development of the human infant in order to study the human visual sensitive period and its time course.

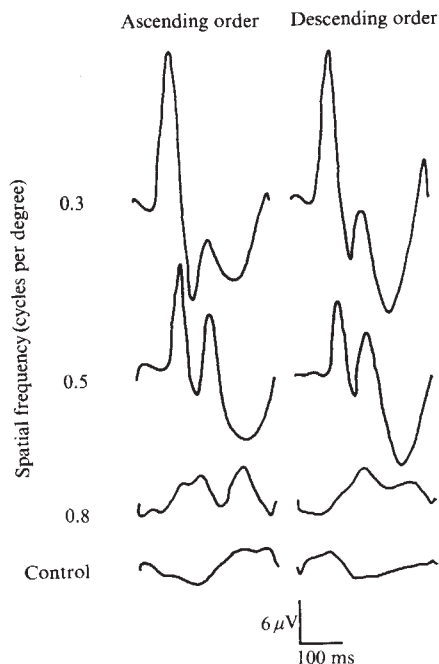
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- Hubel, D. H., and Wiesel, T. N., *J. Neurophysiol.*, **26**, 994-1002 (1963).
- Hubel, D. H., and Wiesel, T. N., *J. Physiol., Lond.*, **206**, 419-436 (1970).
- Hirsch, H. V. B., and Spinelli, D. N., *Science*, **168**, 869-871 (1970).
- Blakemore, C., and Cooper, G., *Nature*, **223**, 477-478 (1970).
- Pettigrew, J. D., and Barlow, H. B., *J. Physiol., Lond.*, **218**, 98-100P (1971).
- Blakemore, C., and Mitchell, D. E., *Nature*, **24**, 467-468 (1973).
- Rose, G. H., Gruneau, S., and Spencer, J. W., *Electroenceph. clin. Neurophysiol.*, **33**, 141-158 (1972).
- Berkley, M. A., and Watkins, D. W., *Vision Res.*, **13**, 403-415 (1973).
- Campbell, F. W., Maffei, L., and Piccolino, M., *J. Physiol., Lond.*, **229**, 719-731 (1973).
- Muir, D. W., and Mitchell, D. E., *Science*, **180**, 420-422 (1973).
- Blake, R. B., Cool, S. J., and Crawford, M. L. J., *Vision Res.*, **14**, 1211-1217 (1974).
- Wässle, H., *Vision Res.*, **11**, 995-1006 (1971).
- Blakemore, C., *Br. med. Bull.*, **30**, 152-157 (1974).





## Functional and neurochemical correlates of potentiation of striatal asymmetry by callosal section

AFTER lesions have been made in one side of the nigro-striatal system, rats have been found to rotate when administered drugs which release dopamine (such as amphetamines) or activate dopamine receptors (such as apomorphine) in the corpus striatum (see for example refs 1 and 2). Because this circling effect is specific to the nigro-striatal system<sup>3</sup> and because it has been established that rats consistently rotate contralateral to the more active striatum (for example ref. 4), the lesion-rotation paradigm is now commonly used as a model for the study of nigro-striatal function. Studies<sup>5,6</sup> in our laboratory have shown that rats without lesions also rotate when administered doses of the same drugs larger than those administered to lesioned rats. As in lesioned rats, the direction of rotation is consistent for normal rats; when tested on several different occasions with the same dose of the same drug, some rats consistently rotate to the right and others rotate to the left. This suggested that normal rats have an intrinsic asymmetry between left and right sides of the nigro-striatal system which is accentuated by drugs. This functional asymmetry corresponds to a chemical asymmetry<sup>7</sup>: the dopamine contents of normal left and right

or right during the pre- and post-injection periods were totalled separately and the net positive rotational difference (that is rotations in the dominant direction minus rotations in the opposite direction) was determined for each rat. Approximately 3 h after testing, six rats were subjected to section of the anterior corpus callosum (from 0.5 mm posterior to bregma to the frontal pole) using procedures developed by Teitelbaum<sup>11</sup>. Sham surgery was performed on the other six rats. One week later, all rats were retested in the rotometer with the same dose of (+)-amphetamine. All rats were then killed and perfused with 10% formalin; brains were removed and immersed in formalin for at least a week, after which sections (40 nm stained with Luxol blue and cresyl violet) were made and histological examination was conducted. In all six rats of the experimental group, the anterior corpus callosum was severed as intended; except for slight cortical damage in one rat, there was no damage to other structures.

Table 1 shows that whereas the sham-operated group made approximately the same number of rotations in both test sessions, the callosal-sectioned group made about twice as many rotations in the second session as in the first. For all 12 rats, the direction of rotation was the same during both sessions. Callosal section, therefore, only potentiated a normal bias.

For the neurochemical experiment, six rats were again subjected to section of the anterior corpus callosum and six received sham surgery. A week after surgery, each rat was killed by decapitation, its brain was removed and the left and right striata were dissected. Each striatum was initially homogenised by a Tissumizer in 4.0 ml of acetonitrile containing 0.2 µg of propionylcholine as an internal standard for analysis of acetylcholine. The homogeniser was washed with an additional 3.0 ml of acetonitrile. Homogenates were combined and centrifuged at 2,500 r.p.m. for 10 min. The acetonitrile phase was removed and assayed for acetylcholine by pyrolysis-gas chromatography<sup>12</sup>. The pellet was resuspended in 5.0 ml of 0.4 N perchloric acid containing 0.1 ml of 10% EDTA, homogenised in a Teflon-glass homogeniser and then centrifuged at 15,000 r.p.m. for 20 min in a refrigerated centrifuge. Striatal dopamine was determined in the extract by a spectrofluorometric method<sup>13</sup>.

Table 2 shows that callosal section had no effect on mean levels of striatal dopamine and acetylcholine. The callosal section, however, enhanced a striatal dopamine asymmetry and induced a striatal acetylcholine asymmetry. In the sham-operated group, there was a significant difference in dopamine content between striata, confirming previous work<sup>7,14</sup>, and no significant difference in striatal acetylcholine content. In the callosal-sectioned group, striatal dopamine asymmetry was approximately twice as great as in the sham-operated group, and an asymmetry in striatal acetylcholine content was significant. Moreover, in the callosal-sectioned group, the striatal acetylcholine asymmetry was in the opposite direction to the striatal dopamine asymmetry, that is, for each rat, the side containing more dopamine had less acetylcholine. In addition, for the latter group, the striatal dopamine asymmetry (H/L ratio) was inversely correlated with the striatal acetyl-

**Table 1** Effect of callosal section on rotation induced by (+)-amphetamine (1.0 mg kg<sup>-1</sup>)

| Group    | Mean net rotations ± s.e. per 1 h |               |
|----------|-----------------------------------|---------------|
|          | Preoperative                      | Postoperative |
| Callosal | 48.2 ± 14.6                       | 97.7 ± 12.2*  |
| Sham     | 46.8 ± 14.5                       | 49.0 ± 15.1   |

\*Significantly different from preoperative value at  $P < 0.01$ , paired  $t$  test.

striata were found to differ by 10–15%. After administration of (+)-amphetamine, the dopamine contents of left and right striata were found to differ by 25%. Moreover, in response to (+)-amphetamine, rats consistently rotated contralateral to the side containing the most dopamine. This normal asymmetry suggests an inter-hemispheric mechanism that maintains or regulates the asymmetry. Mensah and Deadwyler<sup>8</sup> demonstrated in rats a pathway between left and right caudate nuclei coursing through the ventral corpus callosum. It seemed plausible that this pathway may be part of the mechanism maintaining striatal asymmetry. We have now found that the striatal asymmetry is potentiated, both functionally and chemically, by callosal section.

Twelve naive female Sprague-Dawley rats about 3 months old (approximately 250 g) were placed individually in a rotometer modified<sup>9</sup> after that described by Ungerstedt and Arbuthnott<sup>10</sup>. After 15 min each rat was injected intraperitoneally with (+)-amphetamine sulphate (1.0 mg kg<sup>-1</sup>). Rotations were recorded automatically on a printout counter during the 15 min before and 60 min after injection. Rotations to the left

**Table 2** Effects of callosal section on mean unilateral striatal dopamine and acetylcholine levels (µg g<sup>-1</sup>)\*

| Group                         |          | Left        | Right       | High         | Low         | Mean ratio (H/L) |
|-------------------------------|----------|-------------|-------------|--------------|-------------|------------------|
| Striatal dopamine ± s.e.      | Callosal | 6.87 ± 0.51 | 6.57 ± 0.49 | 7.67 ± 0.44† | 5.77 ± 0.43 | 1.33 ± 0.07‡     |
|                               | Sham     | 6.73 ± 0.39 | 6.56 ± 0.53 | 7.11 ± 0.46† | 6.18 ± 0.38 | 1.15 ± 0.04      |
| Striatal acetylcholine ± s.e. | Callosal | 5.13 ± 0.47 | 4.70 ± 0.41 | 5.35 ± 0.40† | 4.48 ± 0.41 | 1.19 ± 0.05‡     |
|                               | Sham     | 4.82 ± 0.32 | 4.65 ± 0.23 | 4.94 ± 0.34  | 4.53 ± 0.25 | 1.08 ± 0.04      |

\*Mean dopamine and acetylcholine were computed in two ways: left side against right side; and side containing higher level against side containing lower level. In addition, the mean high side/low side (H/L) ratio was also computed.

†High side significantly different from low side ( $t$  tests,  $P < 0.05$ – $0.01$ ).

‡Callosal-sectioned significantly different from sham-operated ( $t$  test,  $P < 0.05$ ).

choline asymmetry ( $r = -0.92$ , significant at  $P < 0.01$ ,  $t$  test); there was no significant correlation for the comparable measurements in the sham-operated group ( $r = 0.21$ ).

These results support the initial hypothesis that a callosal pathway serves to regulate or maintain a set degree of balance or imbalance between the two striata. After section of this pathway, the asymmetry is potentiated, both behaviourally and neurochemically. The reciprocal relationship between the dopamine and acetylcholine asymmetries in the callosal-sectioned group is consistent with recent ideas concerning relationships between central cholinergic and dopaminergic mechanisms<sup>15</sup>. If the normal asymmetry in striatal function is related to spatial preferences, as previous studies suggest<sup>14,16</sup>, then different levels of activity in the intercaudate callosal pathway, as affected by reciprocal dopaminergic and cholinergic mechanisms, may be responsible for normal variations in sidedness among different animals.

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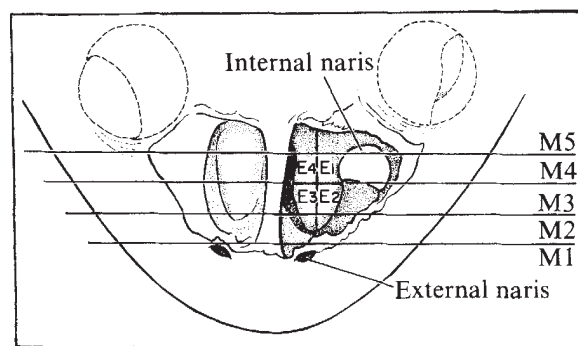
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- <sup>1</sup> Ungerstedt, U., *Acta physiol. scand.*, Suppl. 367, 49 (1971).
- <sup>2</sup> Ungerstedt, U., *Acta physiol. scand.*, Suppl. 367, 69 (1971).
- <sup>3</sup> Crow, T. J., *Expl. Neurol.*, 32, 247 (1971).
- <sup>4</sup> Arbuthnot, G. W., and Crow, T. J., *Expl. Neurol.*, 30, 484 (1971).
- <sup>5</sup> Jerussi, T. P., and Glick, S. D., *Neuropharmacology*, 13, 283 (1974).
- <sup>6</sup> Jerussi, T. P., and Glick, S. D., *Psychopharmacologia*, 40, 329 (1975).
- <sup>7</sup> Glick, S. D., Jerussi, T. P., Waters, D. H., and Green, J. P., *Biochem. Pharmacol.*, 23, 3223 (1974).
- <sup>8</sup> Mensah, P., and Deadwyler, S., *J. Anat.*, 117, 281 (1974).
- <sup>9</sup> Glick, S. D., and Greenstein, S., *Br. J. Pharmacol.*, 49, 316 (1973).
- <sup>10</sup> Ungerstedt, U., and Arbuthnot, G. W., *Brain Res.*, 24, 485 (1970).
- <sup>11</sup> Teitelbaum, H., *J. comp. Physiol. Psychol.*, 76, 51 (1971).
- <sup>12</sup> Szilagyi, P. I. A., Green, J. P., Brown, O. M., and Margolis, S., *J. Neurochem.*, 19, 2555 (1972).
- <sup>13</sup> Lavery, R., and Taylor, K. M., *Analyt. Biochem.*, 22, 269 (1968).
- <sup>14</sup> Zimmerberg, B., Glick, S. D., and Jerussi, T. P., *Science*, 185, 623 (1974).
- <sup>15</sup> Hornykiewicz, O., in *Recent Advances in Parkinson's Disease* (edit. by McDowell, H., and Markham, C. H.), 33 (Davis, Philadelphia, 1971).
- <sup>16</sup> Glick, S. D., and Jerussi, T. P., *J. Pharmac. exp. Ther.*, 188, 714 (1974).

## Distribution of butanol molecules along bullfrog olfactory mucosa

ALTHOUGH electrophysiological<sup>1</sup> and gas chromatographic<sup>2</sup> studies have indicated that odorant molecules drawn into the intact frog olfactory sac establish a concentration gradient along the olfactory mucosa, conclusive evidence for such gradients requires a direct determination of how the molecules are actually distributed. This requirement is met here by labelling butanol molecules with tritium and directly mapping their sorption along the mucosa.

Bullfrogs (*Rana catesbeiana*) anaesthetised with urethane were mounted in a headholder and kept in a hood designed for use with radioactive gases. The concentration (measured in partial pressure) of the tritiated butanol was controlled by a flow dilution olfactometer<sup>1</sup>, from which the tritiated butanol was delivered to the frog's intact olfactory sac through a Teflon cannula placed in the external naris. The stimulus was drawn into the sac as an artificially produced sniff of controlled flow rate. This was accomplished using the negative pressure developed by a Harvard withdrawal pump which was connected to a Teflon cannula placed in the internal naris and secured there by a dental moulding material. The stimulus



**Fig. 1** The relationship of the dorsal surface and eminentia sections to the intact olfactory sac of the bullfrog. Immediately after the stimulus was presented, the frog was quick-frozen in liquid nitrogen. This minimised any further movement of odorant molecules which could have resulted from diffusion and/or desorption. With the animal frozen solid, a No. 3 jeweller's saw was used to remove, in one piece, the dorsal, lateral, and septal walls of the olfactory sac. To further section this piece and at the same time prevent its thawing, the piece was mounted in a specially constructed mitre box which could be maintained at a temperature below 0 °C. In this way the dorsal surface could be sawed into five sections perpendicular to the long axis of the olfactory sac's surface. These five sections were designated M1 to M5 with M1 being the section cut just caudal to the external naris and M4 being that part of the dorsal surface mucosa overhanging the internal naris. When the dorsal, lateral, and septal walls of the olfactory sac were removed, its ventral surface (which contains the eminentia olfactoria) remained attached to the frozen carcass. The eminentia was then cut into four sections designated E1 to E4 and each section was then removed from the animal. All these sections (M1 to M5 and E1 to E4) were individually dissolved in tissue solubiliser and counted in a liquid scintillation system. The number of disintegrations per minute for each mucosal section is an index of the total number of butanol molecules attracted to it. The long black lines running the length of the figure refer to the dorsal surface, which is removed in this drawing. E1 to E4 are indicated by the thicker crossed lines drawn along the surface of the eminentia.

volume was controlled by the duration of the artificially produced sniff since, at a constant flow rate, duration determines volume. Labelled molecules not adhering to the mucosal surface were collected in a toluene trap placed in the Teflon line between the internal naris and the withdrawal pump. The animal was then frozen and sectioned (Fig. 1).

The first column of Table 1 shows the distribution of butanol molecules resulting from the artificially produced sniff in which the partial pressure of the stimulus was 0.36 mmHg; the flow rate 16 ml min<sup>-1</sup>; the duration 1.56 s; and the volume 0.42 ml.

There is an obvious decreasing gradient of butanol molecules from the section which contains the external naris (M1) to that section overhanging the internal naris (M4), and the gradient is rather steep. There is also a gradient, though less steep, rostro-caudally along the eminentia. It is possible, however, that these gradients may simply reflect differences in the surface areas. To correct for these differences, the percentage of the molecules recovered in each section was divided by the mean surface area for that particular section. Correcting for surface area does not reduce these gradients (Table 1); indeed, the dorsal surface gradient is now even steeper.

The comparatively small number of molecules sorbed by the eminentia compared with the dorsal surface is surprising. This is perhaps caused by the poor location of the eminentia for the attraction of chemicals, which, like butanol, are heavily sorbed near the entrance to the olfactory sac, a region into which the eminentia does not even protrude. Such observations raise questions concerning the role of different regions of the mucosa.

If the gradient of odorant molecules along the mucosa has an effect on the processing of information by the olfactory system, the stability of these gradients as the stimulus intensity is varied becomes an important consideration<sup>2</sup>. Therefore, holding volume and flow rate constant at the above levels, we compared



**Table 1** Mucosal distribution of butanol molecules

| Section           | Total molecules recovered (%) | Mean surface area (mm <sup>2</sup> )* | Total molecules recovered (% mm <sup>-2</sup> ) | Mean of all concentrations: % total molecules recovered |
|-------------------|-------------------------------|---------------------------------------|-------------------------------------------------|---------------------------------------------------------|
| Dorsal Surface    |                               |                                       |                                                 |                                                         |
| M1 (most rostral) | 84.38 ± 1.29                  | 4.5 ± 0.1                             | 18.75                                           | 84.59 ± 1.98                                            |
| M2                | 5.81 ± 1.41                   | 14.2 ± 0.3                            | 0.41                                            | 6.46 ± 1.77                                             |
| M3                | 1.86 ± 0.85                   | 19.0 ± 0.5                            | 0.10                                            | 1.60 ± 0.89                                             |
| M4                | 1.00 ± 0.44                   | 21.4 ± 0.6                            | 0.05                                            | 0.84 ± 0.50                                             |
| M5 (most caudal)  | 0.95 ± 0.62                   | 10.7 ± 4.1                            | 0.09                                            | 0.56 ± 0.37                                             |
| Eminentia         |                               |                                       |                                                 |                                                         |
| E2 (rostromedial) | 2.85 ± 0.75                   | 3.7 ± 0.2                             | 0.77                                            | 2.37 ± 0.91                                             |
| E3 (rostromedial) | 2.11 ± 0.58                   | 3.7 ± 0.1                             | 0.57                                            | 2.04 ± 0.95                                             |
| E1 (caudolateral) | 0.42 ± 0.21                   | 4.3 ± 0.2                             | 0.10                                            | 0.33 ± 0.19                                             |
| E4 (caudomedial)  | 0.11 ± 0.05                   | 4.2 ± 0.2                             | 0.03                                            | 0.16 ± 0.11                                             |
| Not Sorbed        | 0.51 ± 0.41                   | —                                     | —                                               | 1.05 ± 0.63                                             |
| Number of animals | 8                             | 4                                     | —                                               | 30                                                      |

\*Means based on four animals (comparable in weight to those used first two columns) which had not received any radioactive stimulus and were frozen and cut as described in the legend to Fig. 1.

Results (%) represent the number of molecules found in each section compared to the total number of molecules recovered. Mean ± s.d. for that particular section taken from eight animals.

the mucosal distribution patterns of butanol molecules established by five different partial pressures: 0.06, 0.36, 0.62, 0.92, and 6.78 mmHg. At least four animals were run at each partial pressure.

Although the absolute number of butanol molecules recovered from each mucosal section increased with partial pressure, the relative number of molecules from section to section remained constant. That is, an analysis of variance showed no significant differences among the percentages of the total number of molecules recovered from each section as a function of partial pressure. Therefore, the results for each partial pressure are not presented individually, but are instead represented by mean values (last column, Table 1).

The fact that butanol establishes a steep concentration gradient and that this gradient remains unchanged throughout a wide range of concentration explains Mozell's earlier electrophysiological data<sup>1</sup>. He showed that over a two log unit range of partial pressures butanol consistently produced measurable responses on the olfactory nerve branch supplying a region of the dorsal mucosa near the external naris, whereas the same butanol concentrations usually produced no measurable response on the nerve branch supplying the mucosal region overhanging the internal naris. Mozell's interpretation of these data, namely, a rather steep mucosal concentration gradient, has now been directly and graphically confirmed by our radioisotope study. Only at the highest partial pressure obtainable at ambient temperature did Mozell observe significant activity on the internal naris nerve branch. In terms of our study this would suggest that the saturated vapour, although still distributed along the mucosa in the same relative proportions, now provides a large enough absolute number of molecules at the M4 region to yield a measurable electrophysiological response.

We do not mean to infer, however, that all odorants will have the same mucosal concentration gradient as butanol. Indeed, from previous chromatographic<sup>2</sup> and electrophysiological<sup>1</sup> studies we predict that odorants with mucosal retention times<sup>2</sup> shorter than butanol, would, in a given sniff, have a less steep gradient. Likewise, those odorants with long retention times might well show a gradient even steeper than that reported here.

In summary, a butanol odorant stimulus results in the establishment of a concentration gradient along the intact olfactory sac and this gradient remains constant within a rather broad range of stimulus intensities. This is what Mozell<sup>2</sup> predicted was necessary if a 'chromatographic-like' differentiation were one of the mechanisms basic to odorant discrimination. Even if these distributions are not basic to olfactory discrimination themselves, they could be fundamental to any process which actually underlies olfactory discrimination. For example, selectively tuned receptors along the flow path would be reached by different concentrations of the same odorant. Therefore, this

initial distribution of the molecules along the olfactory receptor sheet is likely to be of central importance to our full understanding of both quality and intensity discrimination.

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<sup>1</sup> Mozell, M. M., *J. gen. Physiol.*, **50**, 25–41 (1966); *ibid.*, **56**, 46–63 (1970).

<sup>2</sup> Mozell, M. M., and Jagodowicz, M., *Science*, **181**, 1247–1249 (1973); *Ann. N. Y. Acad. Sci.*, **237**, 76–90 (1974).

## Triethylcholine as a precursor to a cholinergic false transmitter

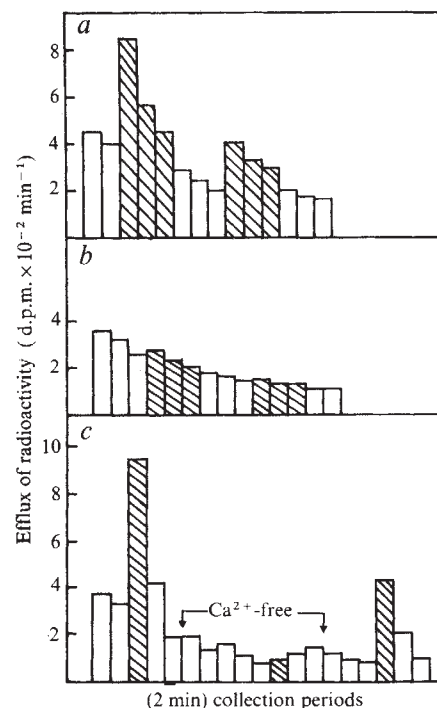
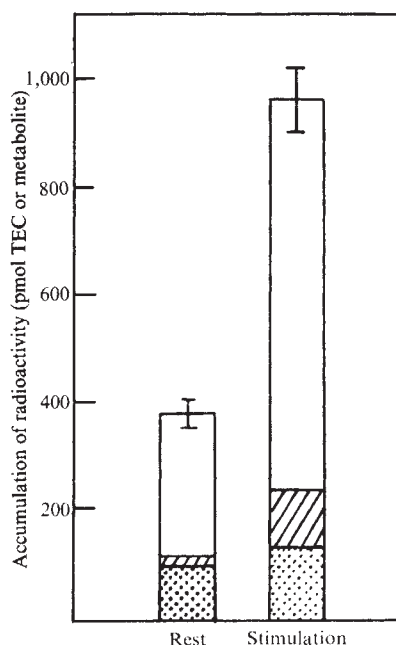
A FALSE transmitter is a substance that is stored and released by a nerve terminal instead of its normal transmitter. Several false transmitters have been demonstrated at adrenergic synapses<sup>1</sup>, but there has been no report of the synthesis, storage and release of a false transmitter at cholinergic synapses. It has been suggested that the triethyl analogue of choline (2-hydroxyethyl-triethylammonium iodide, TEC) might form a cholinergic false transmitter; *in vitro* studies have shown that it can be accumulated by tissues that take up choline<sup>2</sup>, and that choline acetyltransferase can acetylate TEC (refs 3 and 4). Nothing is known, however, about the specificity of the processes associated with storage and release of acetylcholine in intact tissue. The possibility that TEC can form a false transmitter was studied in the present experiments. The results demonstrate the uptake of TEC, its acetylation, and the release of acetyl-TEC by the superior cervical ganglion of the cat.

To measure the uptake and metabolism of TEC, ganglia were perfused<sup>5,6</sup> for 60 min with Krebs solution (mM: NaCl, 120; KCl, 4.6; CaCl<sub>2</sub>, 2.4; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.2; glucose, 9.9) containing <sup>14</sup>C-TEC (*N*-ethyl labelled; 8.6 mCi mmol<sup>-1</sup>; 10<sup>-5</sup> M); in some experiments the preganglionic nerve was stimulated throughout (20 Hz, 0.3 ms, supramaximal voltage). Ganglia were removed, extracted with trichloroacetic acid (TCA; 10% w/v), and the TCA was removed with ether (initial experiments showed that TCA extracted almost all of the radioactivity accumulated by ganglia; <5% was extracted with phospholipids). The fate of TEC accumulated by ganglia was determined by shaking the aqueous

extract with tetraphenylboron in heptanone<sup>7</sup> (10 mg ml<sup>-1</sup>); phosphoryl-TEC remained in the aqueous layer, but acetyl-TEC, and unchanged TEC were extracted into the organic phase from which they were recovered into hydrochloric acid (1 N; HCl). The HCl was removed by evaporation under reduced pressure, and the residue was incubated (30 °C for 30 min) with choline kinase (prepared from brewer's yeast; incubation mixture contained choline kinase 3 mg per ml protein, and (mM): ATP, 8.3; MgCl<sub>2</sub>, 12.5; glycyl-glycine, 83, pH 8.5); TEC, but not acetyl-TEC, was converted to phosphoryl-TEC which was separated from the acetyl-TEC by extracting the latter into heptanone containing tetraphenylboron. The breakdown of acetyl-TEC during these procedures was <5%. In stimulated ganglia, 13.7 ± 1.3% (mean ± s.e.) of the accumulated radioactivity was phosphoryl-TEC, 10.6 ± 2.2% (102 pmol) was acetyl-TEC, and the rest was unchanged TEC. The identity of these metabolites was confirmed by thin-layer chromatography. In non-stimulated ganglia, total uptake of radioactivity was only 41% of that by stimulated ganglia (Fig. 1), and less than 4% (<15 pmol) of the accumulated radioactivity was acetyl-TEC. Thus, nerve stimulation increased the synthesis of acetyl-TEC, and also increased the uptake of TEC. This extra TEC also accumulated in stimulated ganglia when synaptic transmission was blocked by tubocurarine (3 × 10<sup>-5</sup> M) and therefore, it probably entered preganglionic terminals.

In 13 experiments, ganglia were perfused first with <sup>14</sup>C-TEC during preganglionic nerve stimulation (60 min), stimulation was then stopped, and perfusion was continued with TEC-free medium containing eserine sulphate (3 × 10<sup>-5</sup> M); after washing out the radioactivity for 30 min, the preganglionic nerve was again stimulated to test the release of accumulated radioactivity. In all experiments, nerve stimulation increased the release of radioactivity from ganglia (Fig. 2a). In other experiments, the ganglion was not stimulated during exposure to TEC; little, if any, radioactivity was released from these ganglia during subsequent nerve stimulation (Fig. 2b). This suggested that acetyl-TEC, not unchanged TEC, was released by nerve stimulation, and this was confirmed by treating the samples collected before and during nerve stimulation with choline kinase (see above). Radioactivity collected at rest was un-

**Fig. 1** Accumulation of radioactivity by cat superior cervical ganglia. Ganglia were perfused (60 min) with Krebs solution containing <sup>14</sup>C-TEC (10<sup>-5</sup> M) at rest or during stimulation (20 Hz) of the preganglionic nerve. Unshaded columns represent unchanged TEC pmol; mean ± s.e., hatched areas represent acetyl-TEC, stippled areas represent phosphoryl-TEC.



**Fig. 2** The effect of preganglionic nerve stimulation (20 Hz during collection periods indicated by the hatched columns) on the efflux of radioactivity from cat superior cervical ganglia perfused with Krebs solution containing eserine (3 × 10<sup>-5</sup> M). The ganglia had previously been perfused for 60 min with Krebs solution containing <sup>14</sup>C-TEC (10<sup>-5</sup> M) during preganglionic nerve stimulation (a and c) or during rest (b), and then (all experiments) for 30 min (no stimulation) with TEC-free medium containing eserine. In (c) perfusion was switched to a calcium-free medium (containing 10<sup>-4</sup> M EGTA) during the time indicated by the arrows.

changed TEC, and the extra radioactivity released during nerve stimulation was all acetyl-TEC. The amount of acetyl-TEC released at the onset of the test period of stimulation was 19.1 ± 2.9 pmol min<sup>-1</sup> (estimated from the extra radioactivity collected in the first 2 min of stimulation); this represents the release per minute of about 19% of the ganglionic content of acetyl-TEC, which suggests that acetyl-TEC is at least as available for release by nerve impulses as is acetylcholine under similar experimental conditions<sup>8</sup>. The release of acetylcholine from nerve terminals is known to depend on the presence of calcium, and in two experiments, the release of acetyl-TEC by nerve stimulation was tested before, during and after perfusion with a calcium-free medium (containing 10<sup>-4</sup> M EGTA). The release of acetyl-TEC was calcium-dependent (Fig. 2c).

The present experiments show that TEC is taken up by cholinergic nerve terminals, that it is acetylated, and that acetyl-TEC can be released by nerve impulses in a manner similar to the release of the normal neurotransmitter. Acetyl-TEC is pharmacologically inactive on cholinergic receptors<sup>9</sup>; TEC, therefore, forms a false but inactive cholinergic transmitter.

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- Kopin, I. J., *A. Rev. Pharmac.*, **8**, 377-394 (1968).
- Hemsworth, B. A., and Bosmann, H. B., *Eur. J. Pharmac.*, **16**, 164-170 (1971).
- Burgen, A. S. V., Burke, G., and Desbarats-Schonbaum, M.-L., *Br. J. Pharmac. Chemother.*, **11**, 308-312 (1956).
- Hemsworth, B. A., and Smith, J. C., *J. Neurochem.*, **17**, 171-177 (1970).
- Kibjakow, A. W., *Pflügers Archs ges. Physiol.*, **232**, 432-443 (1933).



- <sup>6</sup> Collier, B., and Lang, C., *Can. J. Physiol. Pharmacol.*, **47**, 119–126 (1969).  
<sup>7</sup> Fonnum, F., *Biochem. J.*, **113**, 291–298 (1969).  
<sup>8</sup> Birks, R. I., and MacIntosh, F. C., *Can. J. Biochem. Physiol.*, **39**, 787–827 (1961).  
<sup>9</sup> Holton, P., and Ing, H. R., *Br. J. Pharmacol. Chemother.*, **4**, 190–196 (1949).

## Immunoreactive thyrotrophin releasing factor in gastropod circumoesophageal ganglia

REPORTS have indicated that thyrotrophin releasing factor (TRF) is stored in extrahypothalamic as well as hypothalamic brain regions of many vertebrates from the primitive larval lamprey to the more advanced mammals<sup>1–4</sup>. Although TRF is found in the brain of many poikilotherms, administration of synthetic TRF (*p*Glu-His-ProNH<sub>2</sub>) to these animals does not activate thyroid gland function<sup>5–7</sup>. Thus, it has been proposed that, in these animals, TRF modulates synaptic transmission rather than releasing thyrotrophin (thyroid stimulating hormone)<sup>5</sup>. Furthermore, the administration of TRF to hypophysectomised rodents potentiates the effect of the L-dopa on behaviour<sup>8</sup>, thus supporting the hypothesis that TRF modulates monoaminergic transmission in higher vertebrates as well. Further support for a role of TRF in synaptic transmission is the finding that administration of the synthetic tripeptide leads to an increase in noradrenaline turnover in rat brain<sup>9–11</sup>. Because these reports imply first, that TRF may influence monoaminergic transmission and second, that, in lower vertebrates, this role of TRF may be more important than that of regulating TSH release, we have investigated whether TRF is present in invertebrates which do not produce thyroid hormones but exhibit monoaminergic neurotransmission. We found immunoreactive TRF in the circumoesophageal ganglia of various gastropods. Species studied were the landsnail *Mesodon roemeri* (Connecticut Valley Biological Supply Co., Massachusetts), *Planorbis corneus* (Mogul Ed Corp., Wisconsin), *Helesoma trivolbis* and *Viviparus malleatus* (Ann Arbor Biological Center, Michigan).

The circumoesophageal ganglia were removed and freed of excess connective tissue under a dissecting microscope. The ganglia were frozen immediately and tissues from several animals were pooled. After thawing, they were homogenised in 100  $\mu$ l of distilled water, and proteins were precipitated with 1.0 ml of absolute methanol. After refrigeration overnight, the homogenate was centrifuged at 15,000g for 5 min and the protein concentration of the pellet was determined according to the technique of Lowry *et al.*<sup>12</sup>. The methanolic phase was dried under nitrogen and resuspended in phosphate buffer for radioimmunoassay of TRF. The presence of immunoreactive TRF was determined by the method of Jackson and Reichlin<sup>1</sup> or by a modification of the method of Jeffcoate *et al.*<sup>13</sup>, to be described in detail elsewhere. Both assays showed minimal crossreactivities with TRF breakdown products and structurally related small peptides. The TRF determination in both assays, although carried out with different antibodies, were in good agreement (for example, 125 pg mg<sup>-1</sup> protein compared with 150 pg mg<sup>-1</sup> protein for a pool of ganglia).

**Table 1** Immunoreactive TRF in various gastropods

| Gastropod species          | pg TRF mg <sup>-1</sup> protein |
|----------------------------|---------------------------------|
| <i>Viviparus malleatus</i> | 31.5 (23–40)*                   |
| <i>Helesoma trivolbis</i>  | 65.0†                           |
| <i>Planorbis corneus</i>   | 37.0†                           |
| <i>Mesodon roemeri</i>     | 308.0 (75–720)‡                 |

\*Mean of two pools of ganglia; the range is indicated in parentheses.

†Determination of a single pool of ganglia.

‡Mean of four pools of ganglia; the range is indicated in parentheses.

Table 1 shows that TRF was present in various gastropods from the most primitive species examined (*V. malleatus*) to the more advanced pulmonates (*H. trivolbis*, *P. corneus*, *M. roemeri*). There was more TRF in circumoesophageal ganglia from the landsnail *Mesodon roemeri* than that in those from water snails (mean value of 308 pg mg<sup>-1</sup> protein compared with a pooled mean value of 44.5 pg mg<sup>-1</sup> protein). The amount of TRF in pooled snail ganglia was less than has been reported for hypothalamic tissue of various vertebrates<sup>1</sup>, but was within the range of values reported for cerebral cortical tissues (20 pg mg<sup>-1</sup> protein in the rat to 4,500 pg mg<sup>-1</sup> protein in the tadpole<sup>1</sup>). Although not shown in Table 1, immunoreactive TRF was present in extracts from *Mesodon roemeri* after the methanolic extracts from the circumoesophageal ganglia had been purified on a column of Sephadex SP C-25 according to the method of McKelvy<sup>13</sup>. This indicates that the immunoreactive material found in crude methanolic extracts from these animals is the tripeptide *p*Glu-His-ProNH<sub>2</sub>. In further support of the identity of the immunoreactive material as TRF is the fact that the immunoreactive material from snail ganglia and synthetic TRF showed parallel dilution curves in the radioimmunoassay systems used.

Thus immunoreactive TRF is present in various gastropod species. This supports the evidence obtained from mammals that this molecule has a role in modulating neurotransmission, and suggests that this function evolved before that of controlling the release of thyroid stimulating hormone.

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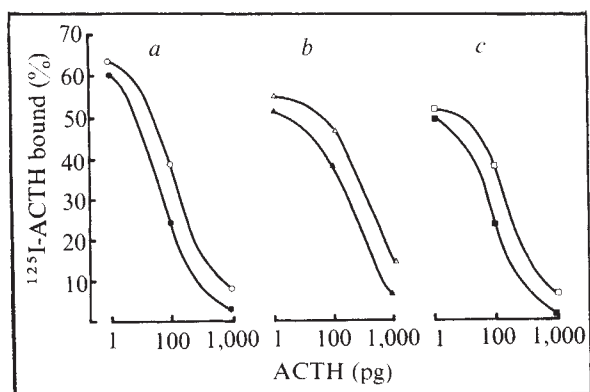
- Jackson, I. M. D., and Reichlin, S., *Endocrinology*, **95**, 816–824 (1974).
- Oliver, C., Eskay, R. L., Ben-Jonathan, N., and Porter, J. C., *Endocrinology*, **95**, 540–546 (1974).
- Jackson, I. M. D., and Reichlin, S., *Life Sci.*, **14**, 2259–2266 (1974).
- Grimm-Jørgensen, Y., and McKelvy, J. F., *J. Neurochem.*, **23**, 471–478 (1974).
- Gorbman, A., and Hyder, M., *Gen. comp. Endocr.*, **20**, 588–589 (1973).
- Vandesande, F., and Aspelagh, M. R., *Gen. comp. Endocr.*, **23**, 355–356 (1974).
- Gona, A. G., and Gona, O., *Gen. comp. Endocr.*, **24**, 223–225 (1974).
- Plotnikoff, N. P., Prange, A. F. Jr., Breese, G. R., Anderson, M. S., and Wilson, I. C., *Science*, **178**, 417–418 (1972).
- Keller, H. H., Bartholini, G., and Pletschner, A., *Nature*, **248**, 528–529 (1974).
- Horst, W. D., and Spirit, N., *Life Sci.*, **15**, 1073–1082 (1974).
- Constantinidis, J., Geissbuhler, F., Gaillard, J. M., Havaguimian, T., and Tissot, R., *Experientia*, **30**, 1182–1183 (1974).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, **193**, 265–275 (1951).
- Jeffcoate, S. L., Fraser, H. M., Gunn, A., and White, N., *J. Endocr.*, **59**, 191–192 (1973).
- McKelvy, J. F., *Analyt. Biochem.* (in the press).

## Possible placental origin of ACTH in normal human pregnancy

It is well known that the free fraction of plasma cortisol is increased in pregnancy<sup>1</sup>, but it has not been established whether this is a result of maternal pituitary or placental adrenocorticotrophic hormone (ACTH) secretion. Maternal plasma ACTH levels in human pregnancy have been variously reported as elevated<sup>2</sup>, or depressed<sup>3</sup>; but there is no information on the relationship between ACTH levels and the stage of gestation. We now report that maternal ACTH levels increase progressively throughout pregnancy, that urinary free cortisol levels are raised and show resistance to suppression by dexamethasone

and we present evidence suggesting that ACTH may be produced by the placenta.

A total of 82 heparinised blood samples were collected between 0900 and 1100 from normal, pregnant women attending an antenatal clinic. All samples were separated immediately at 4 °C and stored at -20 °C until assayed and all measurements were made within three weeks of sample collection. Plasma ACTH was determined by radioimmunoassay after extraction on to porous glass<sup>4</sup>. Highly purified human ACTH was used as standard and for iodination. Three antisera were used; one directed against the N-terminal part of the biologically active amino acids 1-24 of the ACTH molecule (extended N-terminal antiserum); a second specific for amino acids 13-18 of the molecule (middle N-terminal antiserum); and the third against the biologically inactive C-terminus (C-terminal antiserum). The specificities of these antisera have been described in detail elsewhere<sup>4-6</sup>. Only the extended N-terminal antiserum was used to measure plasma ACTH; all three were used to measure ACTH in placental extracts.



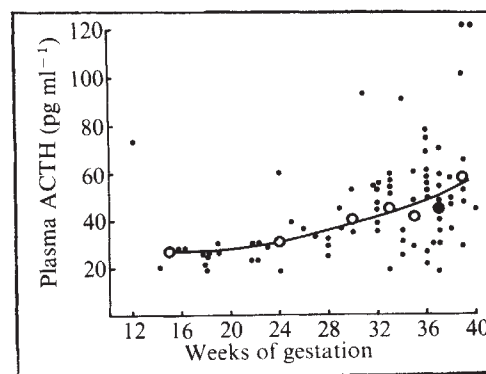
**Fig. 1** Serial dilutions of a placental extract compared with dilutions of standard purified human ACTH using <sup>125</sup>I-ACTH, and N-terminal antiserum (a), and middle N-terminal antiserum (b), and C-terminal antiserum (c). ●, ▲, ■, standards; ○, △, □, placental extracts.

Freshly collected placentas from normal deliveries were trimmed of cord and membranes and washed with 8 l of ice cold 4% saline. Approximately 100 g of tissue was extracted in HCl-acetone (2.5:97.5 v/v)<sup>7</sup>; ACTH was determined by radioimmunoassay in serial dilutions of these extracts. In one extract, ACTH levels were also determined by cytochemical bioassay<sup>8</sup>.

Excretion of free cortisol in the urine was measured by a competitive protein binding assay<sup>9</sup>. The assay was modified for pregnancy urine by the addition of a preliminary wash with an equal volume of petroleum ether to remove progesterone which otherwise interferes in the assay. This procedure removes 95% of <sup>14</sup>C-progesterone, but only 2% of the <sup>3</sup>H-cortisol<sup>10</sup>. Determinations were made in 24 normal women, in 16 patients with pituitary-dependent Cushing's syndrome and in six women during the last trimester of normal pregnancy before and after the oral administration of dexamethasone (2 mg d<sup>-1</sup> for 3 d).

**Table 1** ACTH concentrations in human placentas measured by two radioimmunoassays

| Extended N-terminal antiserum (ng per g wet weight) | C-terminal antiserum (ng per g wet weight) | Bioassay (ng per g wet weight) |
|-----------------------------------------------------|--------------------------------------------|--------------------------------|
| 3.2                                                 | 2.8                                        | 0.66                           |
| 11.3                                                | 16.7                                       |                                |
| 1.2                                                 | 1.0                                        |                                |
| 21.3                                                | 13.1                                       |                                |



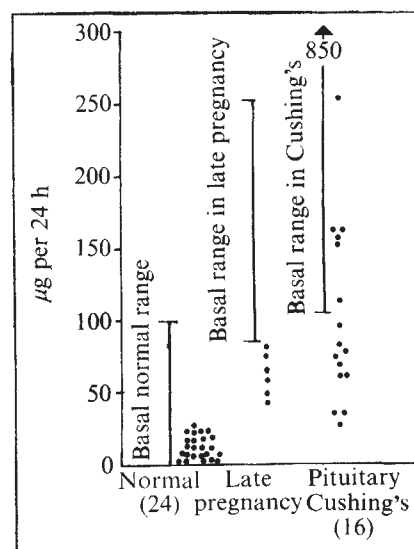
**Fig. 2** Maternal plasma ACTH levels in normal pregnancy. Individual levels are shown (●) together with the mean levels (○) estimated after logarithmic transformation of grouped data (10-20 weeks; 21-27 weeks; 28-31 weeks; 32-33 weeks; 34-35 weeks; 36-37 weeks; 38-40 weeks). (Note the occurrence of a few very high levels which may have been caused by unrecognised stress during sample collection.)

Plasma and placental extracts gave inhibition parallel to that of the standard (Fig. 1). There was a progressive rise in plasma ACTH levels throughout pregnancy (Fig. 2), although most lay within the normal range (<10-80 pg ml<sup>-1</sup>). The placental content of ACTH ranged from 1.0 to 21.3 ng per g wet weight tissue (Table 1).

Following dexamethasone, normal subjects showed suppression of urinary free cortisol to less than 30 µg d<sup>-1</sup> (Fig. 3). Pregnant women, however, showed resistance to suppression; urinary free cortisol levels during dexamethasone administration were similar to those observed in some with pituitary-dependent Cushing's disease.

These results agree with those of Genazzani and his colleagues<sup>2</sup>, in showing elevated plasma ACTH levels during normal pregnancy, although, in contrast to these workers, the ACTH in maternal plasma shows parallel inhibition in our radioimmunoassay. We have also shown a progressive rise in circulating ACTH concentrations throughout the second and third trimesters. The discrepancy between these results and those of Mukherjee and Swyer<sup>3</sup>, who found decreased levels, may be attributed to technical factors, since they did not use a plasma extraction step. Other observations on maternal ACTH levels in human pregnancy cannot be strictly compared with

**Fig. 3** Urinary free cortisol levels before (vertical bars) and after (●) dexamethasone administration in normal women, women in the last trimester of pregnancy and patients with Cushing's disease.





the findings discussed here because the samples were collected at the time of Caesarean section<sup>11</sup> or vaginal delivery<sup>12,13</sup>.

The source of the increasing plasma ACTH levels may be the placenta or the maternal pituitary gland. We have shown that urinary free cortisol levels in women in late pregnancy are resistant to dexamethasone suppression, a situation comparable with that observed in Cushing's disease. This suggests that a component of maternal ACTH in pregnancy is not of pituitary origin. The present studies, in agreement with those of Genazzani<sup>2</sup>, and others<sup>14-16</sup> demonstrate high concentrations of ACTH in the human placenta. Although content does not necessarily reflect either synthesis or release, these levels are comparable with those observed in tumours associated with ectopic ACTH secretion<sup>17</sup> and are much higher than could be accounted for by the content of sequestered blood. This observation, together with the progressive increase in circulating levels during pregnancy and the elevated urinary free cortisol concentrations, suggests placental release of ACTH which is autonomous and not subject to feedback control.

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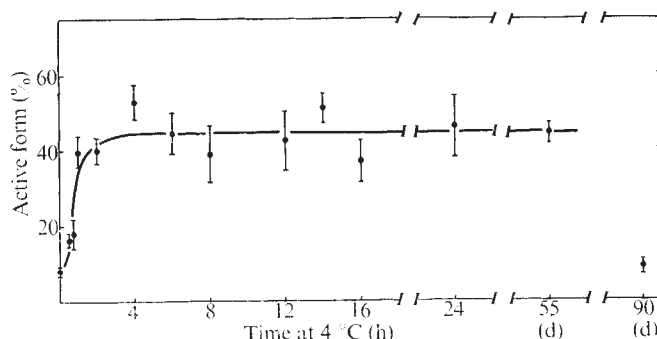
- <sup>1</sup> Burke, C. W., and Roulet, F., *Br. Med. J.*, **i**, 657-659 (1970).
- <sup>2</sup> Genazzani, A. R., Fraioli, F., Hurlimann, J., Felber, J.-P., and Fioretti, P., *Clin. Endocr.*, **4**, 1 (1975).
- <sup>3</sup> Mukherjee, K., and Swyer, G. I. M., *J. Obstet. Gynaecol. Br. Commonw.*, **79**, 504-512 (1972).
- <sup>4</sup> Rees, L. H., et al., *Endocrinology*, **89**, 254-261 (1971).
- <sup>5</sup> Orth, D. N., in: *Methods of Hormone Radioimmunoassay*, (edit by Jaffe, B. M., and Behrman, H. R.), 140 (Academic, New York and London, 1974).
- <sup>6</sup> Ratcliffe, J. G., Knight, R. A., Besser, G. M., Landon, J., and Stanfield, A. G., *Clin. Endocr.*, **1**, 27-44 (1972).
- <sup>7</sup> Lyons, W. R., *Proc. soc. exp. biol. Med.*, **35**, 645 (1937).
- <sup>8</sup> Chayen, J., Loveridge, N., and Daly, J. R., *Clin. Endocr.*, **1**, 219-233 (1972).
- <sup>9</sup> Beardwell, C. G., Burke, C. W., and Cope, C. L., *J. Endocr.*, **42**, 79 (1968).
- <sup>10</sup> Galvao-Teles, A., and Burke, C. W., *Lancet*, **i**, 737-740 (1973).
- <sup>11</sup> Simmer, H. H., et al., *Am. J. Obstet. Gynecol.*, **119**, 283-296 (1974).
- <sup>12</sup> Allen, J. P., Cook, D. M., Kendall, J. W., and McGilvra, R., *J. clin. Endocr. Metab.*, **37**, 230-234 (1973).
- <sup>13</sup> Dokumov, S. I., Milanov, S. C., and Trepetshov, S. P., *J. Obstet. Gynaec. Br. Commonw.*, **81**, 220-221 (1974).
- <sup>14</sup> Jailer, J. W., and Knowlton, A. I., *J. clin. Invest.*, **29**, 1430-1436 (1950).
- <sup>15</sup> Opsahl, J. C., and Long, C. N. H., *Yale J. Biol. Med.*, **24**, 119-209 (1951).
- <sup>16</sup> Lundin, P. M., and Holmdahl, S., *Acta Endocr., Copenh.*, **25**, 388-394 (1957).
- <sup>17</sup> Rees, L. H., and Ratcliffe, J. G., *Clin. Endocr.*, **3**, 263-299 (1974).

## Phosphorylase and glycerol production activated by cold in diapausing silkworm pupae

THE accumulation of glycerol in overwintering insects, first shown in eggs of the silkworm, *Bombyx mori*<sup>1</sup>, and pupae of certain saturniid silkworms<sup>2</sup>, is now known in many species. Although not all cold-resistant insects contain high levels of glycerol, and the mechanisms of tolerance to cold are not fully understood, it is accepted that naturally produced glycerol can act as an antifreeze and contribute to protection against cold injury<sup>3-5</sup>.

In certain insects, such as adult carpenter ants (*Camponotus pennsylvanicus*<sup>6</sup>) and Alaskan carabid beetles (*Pterostichus brevicornis*<sup>7</sup>), production of glycerol depends on exposure to cold. In diapausing pupae of the silkworm (*Hyalophora cecropia*) some glycerol is produced at 25 °C, but the

rate of production is greatly increased at 6 °C (ref. 7). Table 1 shows that haemolymph glycerol reaches about 250 mM at 25 °C and 500-600 mM after 1 month at 4 °C. Glycerol is formed chiefly at the expense of glycogen<sup>7</sup>, and enzymes forming an appropriate pathway occur in insect tissue: glycogen phosphorylase<sup>8</sup>, the glycolytic pathway to dihydroxyacetone phosphate,  $\alpha$ -glycerophosphate dehydrogenase<sup>10,11</sup> and  $\alpha$ -glycerophosphatase<sup>12,13</sup>. Accordingly, cold might be expected to activate one or more steps in this sequence. We have now demonstrated this.



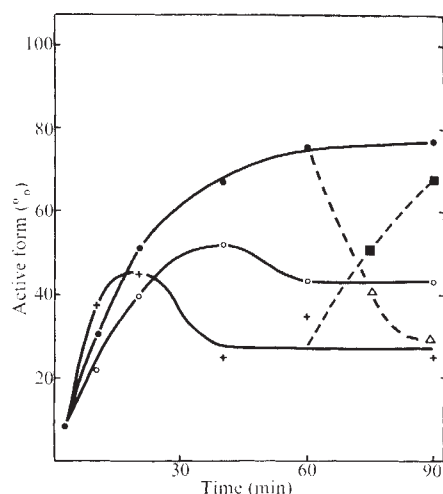
**Fig. 1** Conversion of fat body phosphorylase to the active form after transfer of cecropia pupae from 25 °C to 4 °C. Pieces of fat body were removed within 45 s and frozen between aluminium plates on dry ice. The frozen tissue (about 100 mg) was homogenised in 1.3 ml of buffer (20 mM triethanolamine acetate, pH 7.0, containing 5 mM EDTA and 20 mM NaF). The homogenate was centrifuged twice at 10,000g for 10 min, and 25 or 50  $\mu$ l of the supernatant was used for enzyme assay. A coupled spectrophotometric assay<sup>15</sup> was performed in a Gilford 2400S spectrophotometer. A rate was established in the absence of 5'-AMP, for active phosphorylase, then 2 mM 5'-AMP was added and a new rate was determined for total phosphorylase. Values are means  $\pm$  s.e.m. from 3-10 determinations on individual pupae. The initial level of about 8% active form is higher than that of less than 1% previously found by a different assay method<sup>9</sup>, and recent experiments (L. T. Wimer, R. Z. and G.R.W., unpublished), show a systematic difference between the two assays, the reason for which is not known.

We found in the fat body, which is the site of the major glycogen reserves in diapausing cecropia silkworm pupae, activation of glycogen phosphorylase by cold that seems to play a role in the control of glycerol formation. Phosphorylase in this tissue occurs in active and inactive forms, the latter (like muscle phosphorylase b) being activated by 5'-adenosine monophosphate<sup>9,14</sup>. In fat body excised quickly from pupae stored at 25 °C, usually less than 10% of the phosphorylase was in the active form, but when pupae were placed at 4 °C, the active fraction increased rapidly to about 45% (Fig. 1). After 3 months of storage at 4 °C, this activation had been reversed. Similar effects of temperature were observed with fat body tissue *in vitro* (Fig. 2). When pupal fat body was placed in Ringer solution, phosphorylase was converted partially to the active form

**Table 1** Increase in glycerol content in haemolymph of cecropia silkworm pupae after storage at 4 °C

| Age of pupae (d) | Unchilled (25 °C) | Chilled      |                  |
|------------------|-------------------|--------------|------------------|
|                  | Glycerol (mM)     | Days at 4 °C | Glycerol (mM)    |
| 30               | 147 $\pm$ 9 (5)   | 5.5          | 171 $\pm$ 13 (5) |
| 70               | 278 $\pm$ 35 (3)  | 30           | 490 $\pm$ 80 (3) |
| 90               | 225 $\pm$ 54 (3)  | 40           | 679 $\pm$ 57 (3) |
| 110              |                   | 50           | 512 $\pm$ 50 (4) |
| 110              | 205 $\pm$ 27 (3)  | 60           | 540 $\pm$ 46 (3) |
| 110              |                   | 70           | 438 $\pm$ 45 (3) |

Haemolymph samples were deproteinised with ethanol, and glycerol was determined with chromotropic acid<sup>8</sup>. Means and standard errors, with numbers of samples in parentheses, are shown.



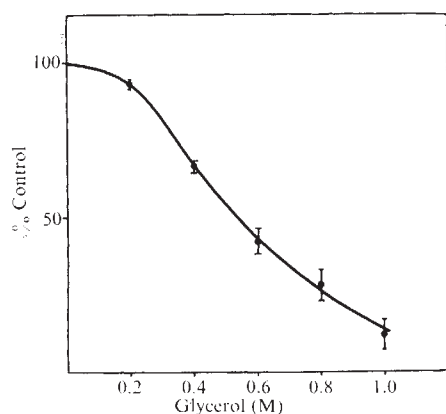
**Fig. 2** Changes in degree of activation of phosphorylase in fat body after incubation of the tissue in Ringer solution at different temperatures. Fat body was removed quickly from diapausing pupae, rinsed very gently and divided into small pieces (about 100 mg), and each was placed in a small beaker with 2 ml of lepidopteran Ringer solution<sup>16</sup> at the temperature shown. At the times indicated, the tissue was frozen between aluminium plates on dry ice. Phosphorylase was then prepared and assayed as described in Fig. 1. ●, 0 °C; ■, 0 °C after transfer; ○, 15 °C; +, 25 °C; △, 25 °C after transfer.

(see ref. 9); at 25 °C this activation was reversed after 20–40 min, but at 0 °C it continued, to produce up to 80% active enzyme at 90 min. Incubation at 15 °C yielded an intermediate curve. Transfer from 25 °C to 0 °C, or from 0 °C to 25 °C, after 60 min showed the effect of temperature to be reversible.

Since the total level of phosphorylase did not change during these experiments, the activity changes probably reflected conversion of enzyme forms by phosphorylase kinase and phosphatase, respectively. Both these activities have been demonstrated in soluble supernatant preparations under appropriate conditions, but in such preparations phosphorylase activation was not enhanced by cold. The possible similar effects in other animal tissues, are being investigated.

The association of changes in phosphorylase activity in cecropia silkworm pupae with glycerol production is sup-

**Fig. 3** Influence of glycerol on the activation of phosphorylase in fat body *in vitro*. Pieces of fat body (about 100 mg) from a single diapausing pupa were incubated in 2 ml of Ringer containing different concentrations of glycerol at 0 °C. After 60 min the fat body was treated and phosphorylase was assayed as described under Fig. 1. The values are expressed as percentage of the increase shown by the control (60 min in Ringer with glycerol). Each value is the mean  $\pm$  s.e.m. of four determinations on different animals.



ported by our observation that the activation of phosphorylase in fat body at 0 °C is inhibited by addition of glycerol to the bathing Ringer solution (Fig. 3). The level of glycerol giving 50% inhibition (about 0.5 M) is often attained in haemolymph *in vivo*. This is suggestive of a regulatory feedback.

These observations indicate that glycogenolysis in diapausing cecropia silkworm pupae is controlled in part by the influence of both environmental temperature and the level of glycerol in the haemolymph on the phosphorylase system. The specific conversion of carbohydrate to glycerol, however, is probably controlled at another step. A regulatory role has been suggested<sup>7</sup> for  $\alpha$ -glycerophosphatase and has recently received support<sup>13</sup>. An effect of cold on this enzymatic step might account for the observation<sup>17</sup> that *Galleria* larvae contained elevated inorganic phosphate and decreased  $\alpha$ -glycerophosphate after chilling at 0 °C.

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- Chino, H., *J. Insect Physiol.*, **2**, 1–12 (1958).
- Wyatt, G. R., and Meyer, W. L., *J. gen. Physiol.*, **42**, 1005–1011 (1959).
- Salt, R. W., *A. Rev. Entomol.*, **6**, 55–74 (1961).
- Asahina, E., *Adv. Insect Physiol.*, **6**, 1–49 (1969).
- Baust, J. G., *Nature new Biol.*, **236**, 219–221 (1972).
- Dubach, P., Smith, F., Pratt, D., and Stewart, C. M., *Nature*, **184**, 288–289 (1959).
- Wyatt, G. R., *Adv. Insect Physiol.*, **4**, 287–360 (1967).
- Burton, M., *Meth. Enzymol.*, **3**, 246–248 (1957).
- Stevenson, F., and Wyatt, G. R., *Archs Biochem. Biophys.*, **108**, 420–429 (1964).
- Chino, H., *J. Insect Physiol.*, **5**, 1–15 (1960).
- Oliver, E. J., Gelb, W. G., Evans, F., Brandts, J. F., and Nordin, J. H., *Cryobiology*, **8**, 465–473 (1971).
- Chino, H., *J. Insect Physiol.*, **6**, 231–240 (1961).
- Jungreis, A. M., *Am. J. Physiol.*, (in the press).
- Wiens, A. W., and Gilbert, L. I., *Comp. Biochem. Physiol.*, **21**, 145–159 (1967).
- Childress, C. C., and Sacktor, B., *J. biol. Chem.*, **245**, 2927–2936 (1970).
- Jungreis, A. M., Jatlow, P., and Wyatt, G. R., *J. Insect Physiol.*, **19**, 225–233, (1973).
- Lenartowicz, E., *Acta biochim Polon.*, **8**, 15–24 (1961).

## Chromatographic demonstration of mineralocorticoid-specific receptors in rat kidney

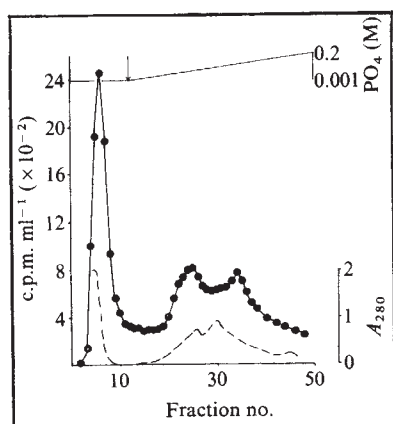
THE binding of a drug with its specific macromolecular receptor(s) seems to constitute the first step whereby a number of pharmacological agents initiate a variety of intra and intercellular processes. Aldosterone is the principal mineralocorticoid in mammals regulating ion transport in the kidney, and some extrarenal targets, but at higher concentrations it also exhibits glucocorticoid-specific properties<sup>1</sup>. From studies on displaceable binding to protein ligands alone, it is not possible to affirm the physicochemical nature and homogeneity of a 'specific' mineralocorticoid receptor that is thought to be essential for the steroid function and said to exist in the toad bladder<sup>2</sup> and rat kidney<sup>3</sup>. Techniques have been established for the characterisation and partial purification of corticosterone receptors in rat liver<sup>4,5</sup>. These have now been adapted to demonstrate aldosterone-specific binding proteins present in rat kidney but absent from the liver.

Figure 1 shows that aldosterone was bound to three sorts of proteins in rat kidney cytosol. The first of these (I) was eluted with the low ionic (0.001 M) prewash on the DE-52 column and seemed to be the most abundant; the second (II) eluted at 0.006 M phosphate and was one-third as radioactive as the first; the third (III) component exhibited nearly the same concentration as the second but came off only at ionic strengths of 0.018 or more. One or more of these could constitute the specific mineralocorticoid receptor(s). When a similar separation

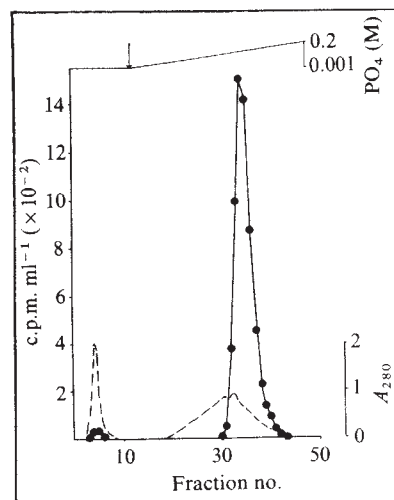


was attempted with kidney cytosol equilibrated at  $10^{-8}$  M corticosterone (Fig. 2) there was only a faint indication of the first peak, the component in the 0.006 M  $\text{PO}_4$  region was totally wanting, whereas nearly all of the radioactivity eluted between 0.02–0.06 M concentration of the phosphate. From a series of other experiments,  $5 \times 10^{-9}$  M aldosterone was actually more effective than a high concentration ( $10^{-7}$  M) of corticosterone in revealing the components that peak in the 0.001 M and 0.006 M  $\text{PO}_4$  regions (although  $10^{-8}$  M aldosterone seemed to be required to saturate these macromolecules). In addition, the elution profile demonstrates that the first peak in the prewash does not represent free radioactivity or an artefact of the washing procedure<sup>8</sup>. Although peaks I and II (Fig. 1) may be explained as polymerised or aggregated forms of the mineralocorticoid receptor, this would be an unlikely postulate for peak III, in view of results shown in Figs 2 and 3.

Mammalian kidney is endowed with gluconeogenic potential and is reported to possess macromolecules with high affinity for glucocorticoids<sup>9</sup>. Peak III (Fig. 1), which has the same elution position as the corticosterone peak in Fig. 2, could therefore represent such receptors, or transcortin, or both. When kidney cytosol equilibrated with  $10^{-7}$  M  $^3\text{H}$ -corticosterone was cochromatographed with  $^{14}\text{C}$ -corticosterone bound to serum transcortin, a shoulder of radioactivity coming off at 0.018–0.02 M was masked by a much larger peak coeluting with serum transcortin at 0.06 M phosphate (Fig. 3). Separation was sharper in the absence of serum and these peaks almost certainly represent the glucocorticoid receptors and transcortin, respectively, whereas peak III (Fig. 1) and the peak in Fig. 2 would seem to be a mixture of both of these components.



**Fig. 1** Ion-exchange separation of aldosterone-binding proteins in rat kidney. Male Wistar rats (150–200 g) were bilaterally adrenalectomised at least 48 h before use, exanguinated under light ether anaesthesia, and perfused with the initial buffer by aortic cannulation. The excised organs were homogenised in an equal volume of the same buffer and the 105,000g supernatant fraction (6 ml) was equilibrated for 60 min at  $4^\circ\text{C}$  with the desired concentration of labelled steroid (aldosterone,  $10^{-8}$  M). The free radioactivity was then removed by additional incubation in presence of 100 mg  $\text{ml}^{-1}$  cell sap of activated charcoal (Sigma C-5260) for 10 min and centrifugation at 3,000g. The supernatant was then passed through glass wool to remove traces of carbon and then charged on to the column. The chromatography on DEAE-cellulose-52 (column  $1 \times 25$  cm) was performed as described previously<sup>4–6</sup>. The column was equilibrated with 0.001 M sodium phosphate, pH 7.5. Aliquots (1 ml) were mixed with 10 ml Unisolve (Koch Light) and counted in a Packard Tricarb Liquid Scintillation Spectrometer with corrections for quenching, spilling and background<sup>7</sup>. After passage of 60–70 ml of the initial buffer (fraction volume 6–7 ml), protein was eluted (at arrow) with a linear gradient of 60 ml 0.001 M and 60 ml 0.2 M Na phosphate, at a flow rate of 60 ml  $\text{h}^{-1}$  (fraction volume approximately 3 ml). All manipulations were carried out at  $4^\circ\text{C}$ .  $1,2,^3\text{H}$ -aldosterone (lot 1172; 55 Ci  $\text{mmol}^{-1}$ ),  $1,2,^3\text{H}$ -corticosterone (batch 57072; 26 Ci  $\text{mM}^{-1}$ ) and  $4,^{14}\text{C}$ -corticosterone (lot 467–248; 57 mCi  $\text{mmol}^{-1}$ ), (Saclay, France) were diluted in ethanol, which was evaporated in the incubation vessel before equilibration. All other chemicals were high purity reagent grade. Dotted line,  $A_{280}$  values; continuous line, radioactivity.

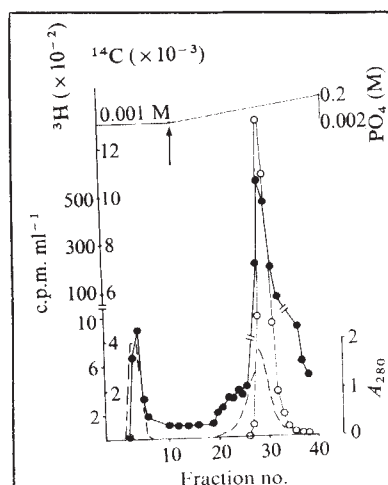


**Fig. 2** Absence of corticosterone binding to mineralocorticoid specific receptors in rat kidney. All details as in legend to Fig. 1 except that the kidney cytosol (5 ml) was equilibrated with  $10^{-8}$  M  $^3\text{H}$ -corticosterone and loaded on the resin.

Since mammalian liver is not the principal site of ion regulation, it is believed to be devoid of mineralocorticoid receptors<sup>1,3</sup>. Figure 4 shows that the 0.006 M region had no radioactivity when liver cytosol equilibrated with  $10^{-7}$  M  $^3\text{H}$ -aldosterone was chromatographed under the conditions described above. Competitive binding, followed by chromatography, further established that liver glucocorticoid receptors elute as a more abundant species in the 0.001 M prewash and a less abundant peak in the 0.018 region of the phosphate gradient (although the latter may also contain some residual, intracellular transcortin) and it is to these macromolecules that aldosterone seems to be bound (Fig. 4).

These results thus provide chromatographic evidence that, as the principal ion regulator, the kidney is endowed with mineralocorticoid-specific receptors which are physicochemically distinct from the glucocorticoid binders, are present as readily distinguishable subpopulations within the cell and are wanting in the liver (having no active role in physiological ion regulation). Furthermore, they seem to be saturated at those endogenous, physiological concentrations at which binding of corticosterone

**Fig. 3** Binding of corticosterone to glucocorticoid receptors and to transcortin. Kidney cytosol (3 ml) and blood serum (2 ml) were saturated with  $10^{-7}$  M  $^3\text{H}$ -corticosterone and  $0.25 \mu\text{Ci}$   $^{14}\text{C}$ -corticosterone, respectively, and then treated with charcoal to remove free radioactivity. After 60 ml prewash with 0.001 M phosphate buffer, elution was carried out (at arrow) with 60 ml each of 0.002 M and 0.2 M phosphate (fraction volume 4 ml) at a flow rate of 60 ml  $\text{h}^{-1}$ . ●  $^3\text{H}$ ; ○  $^{14}\text{C}$ .



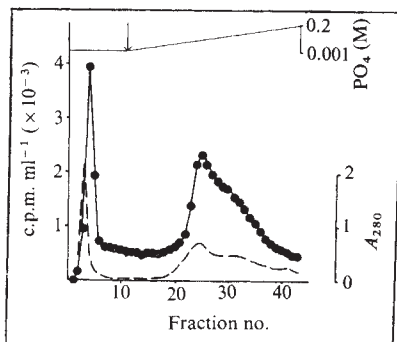


Fig. 4 Absence of mineralocorticoid-specific receptors in rat liver. All details as in legend to Fig. 1 except that liver cytosol (5 ml) was equilibrated with  $10^{-7}$  M  $^3$ H-aldosterone and charged on the column.

is still markedly dose dependent, and this is compatible with the fixed time assay value of  $KD_{37\text{ }^{\circ}\text{C}} 5 \times 10^{-10}$  M (refs 1 and 3). Glucocorticoid specific receptors seem to be identical in the two organs, both with respect to the tenfold higher  $KD$  values and saturation with  $10^{-7}$  M steroid, in keeping with levels circulating *in vivo*<sup>1,3</sup>.

Purification of the receptor proteins is required to establish unequivocal affinity constants. Nevertheless, the techniques described here may profitably be used to understand the mechanism of corticoid hormone action by analysis of receptor function in other organs; the stereospecificity of aldosterone binding in the presence of various agonists and antagonists of the steroid; possible qualitative differences in the relative abundance of the different subpopulations of steroid binders in various stages of differentiation, hypo or hyperfunction; and the relationship between the glucocorticoid and the mineralocorticoid receptors in diverse tissues.

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- <sup>1</sup> Murlow, P. J., and Formann, B. H., *Am. J. Med.*, **53**, 561 (1972).
- <sup>2</sup> Albert, K. G. M. M., and Sharp, G. W. G., *Biochim. biophys. Acta*, **192**, 335 (1969).
- <sup>3</sup> Rousseau, G., Baxter, J. D., Funder, J. W., Edelman, I. S., and Tomkins, G. M., *J. Steroid Biochem.*, **3**, 219 (1972).
- <sup>4</sup> Agarwal, M. K., Shepherd, R. E., and Snart, R. S., *Biochem. J.*, **118**, 5 (1970).
- <sup>5</sup> Snart, R. S., Shepherd, R. E., and Agarwal, M. K., *Hormones*, **3**, 293 (1972).
- <sup>6</sup> Snart, R. S., Sanyal, N. N., and Agarwal, M. K., *J. Endocrinol.*, **47**, 149 (1970).
- <sup>7</sup> Agarwal, M. K., Hanoune, J., Yu, F. L., Weinstein, I. B., and Feigelson, P., *Biochemistry*, **8**, 4806 (1969).
- <sup>8</sup> Beato, M., and Feigelson, P., *J. biol. Chem.*, **247**, 7890 (1972).

## Molecular structure of membrane-bound rhodopsin

WE have results which suggest a structure for the membrane-bound sensory protein, rhodopsin. This structure has novel features which are of considerable biological interest.

The transduction of light energy into neural signals is mediated in all known visual systems by a common type of visual pigment consisting of the 11-*cis* isomer of a retinaldehyde chromophore conjugated to some form of the protein opsin. Photoisomerisation of a single molecule of the photopigment in a vertebrate photoreceptor cell can initiate a chain of events leading to excitation of that cell. Although the primary events of the sequence are not known, it is widely suspected that they include a change in photopigment structure that causes release of some transmitter substance<sup>1-3</sup>.

We looked for structure changes in rhodopsin-containing disk membranes using hydrogen-tritium exchange techniques<sup>4,5</sup>.

Evidence for structure change has previously been limited to rhodopsin in detergent solutions<sup>6-10</sup> and some of these changes have been specifically shown not to occur *in situ*<sup>10,11</sup>. Figure 1 shows that changes do occur in the membrane-bound protein following illumination. The data in Fig. 1 measure only the slowest 25% of the exchanging protein hydrogens.

To study the much larger fraction of protein hydrogens that exchange more rapidly, different exchange conditions must be used. Figure 2 shows data obtained at lower pH where the intrinsic exchange rate of peptide hydrogens is 250 times slower. Disk membranes from cattle and frog gave similar results. While about 0.4 hydrogens per peptide group exchange rather slowly and trace out a series of slower and slower rates, a larger number, ~ 0.7 hydrogens, form a clearly distinguishable and much faster kinetic class. Surprisingly, the exchange rate of the fast phase is experimentally identical to that previously found for peptide group hydrogens that are freely exposed to bulk solvent water.

The factors controlling the hydrogen exchange behaviour of free peptides have been calibrated in studies with small molecules<sup>12</sup> and homopolypeptides<sup>13</sup>. Peptide exchange rates are a strong function of pH and temperature and vary to a minor extent depending on nearest neighbour side chains. The calibrations obtained for these factors have been tested against hydrogen exchange data for the free peptides in a random chain heteropolypeptide, oxidised ribonuclease<sup>12</sup>; in the globular protein, myoglobin<sup>12,14</sup>; in the fibrous protein, collagen<sup>15</sup>; and, by use of nuclear magnetic resonance techniques, for specific protons in the cyclic oligopeptide, angiotensin<sup>16</sup>. In all these cases, the free peptide rates were well behaved and precisely predictable. Under the conditions of Fig. 2, free peptides are expected to have half times ranging generally from 30 to 60 s, and this is found for the fast phase in Fig. 2.

The possibility that protons from other sources might contribute significantly to these curves was considered in some detail. The data in Fig. 2 measure a total of  $1.1 \pm 0.1$  exchanging hydrogens per peptide group. This corresponds closely to the  $1.12 \pm 0.17$  peptide and primary amide hydrogens expected on the basis of composition data for rod outer segment membranes<sup>17-20</sup>. Moreover, experiments were carried out which excluded protons from phospholipids and protein side chains. (These results will be detailed elsewhere.) Thus the data in Figs 1 and 2 represent protein hydrogens.

The data show that close to two-thirds of the peptide hydrogens in disk membranes are hydrogen bonded to solvent water. Most of these must come from rhodopsin itself, since rhodopsin accounted for ~ 85% of the protein in our preparations, in agreement with the value established for uncontaminated rod outer segments<sup>21-23</sup>. Thus we conclude that at least 60% of rhodopsin's peptide hydrogens are freely exposed and hydrogen bonded to water. This seems especially remarkable when it is considered that rhodopsin is an intrinsic membrane protein, and also that the more familiar water soluble proteins that have been studied by either hydrogen exchange or X-ray diffraction or both all have only  $30 \pm 10\%$  of their peptide groups hydrogen bonded to solvent water. Some results for other membrane systems also indicate that their proteins, like typical soluble proteins, have only a small fraction of fast, freely exposed peptides (see ref. 24 for sarcoplasmic reticulum and unpublished results of G. Gabor and S.W.E. for erythrocyte ghosts).

Rhodopsin's structure then must be quite unusual. In addition to the large number of freely exposed peptide groups, a second restriction on possible structural models for rhodopsin is provided by its large complement of apolar residues. Many of these are presumed to be in contact with the lipid hydrocarbon chains since the protein can be separated from the membrane only through the use of detergents. The general, non-committal resolution of these apparently contradictory requirements seems to be that a good part of rhodopsin's polypeptide chain must be arranged at a lipid-water interface with many of its apolar side chains contacting lipid hydrocarbons and many of its peptide group protons in water.

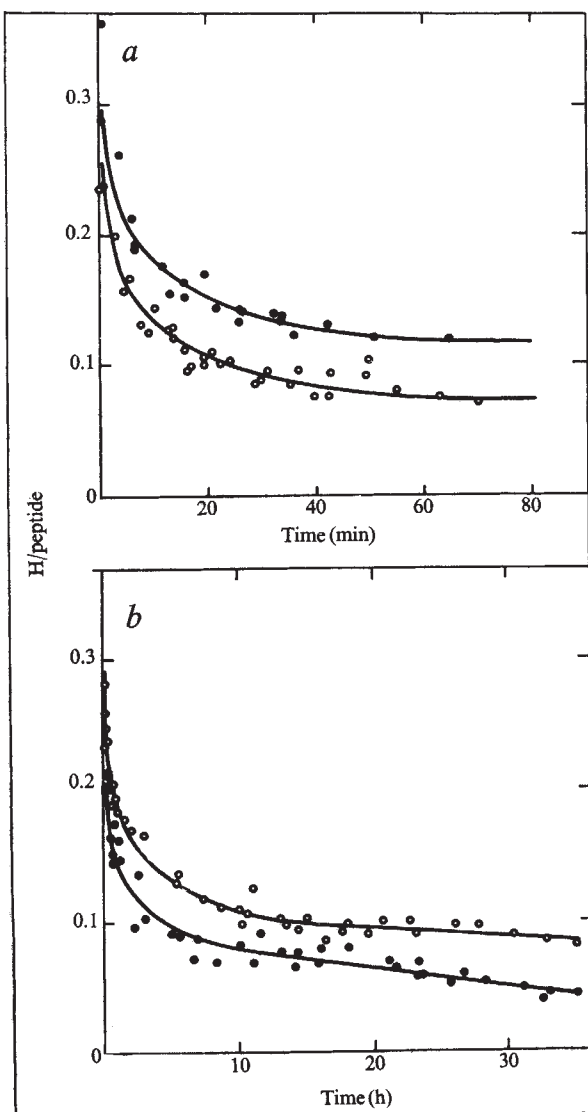


Several considerations argue against the surface of the membrane bilayer. This surface could not provide the required apolar contacts; the highly polar phospholipid head groups are there. Rhodopsin is thought to penetrate into and perhaps through the membrane, as suggested by fluorescence transfer experiments<sup>25</sup>, by the pattern of protease sensitivity of the membrane-bound protein<sup>26,27</sup> and by freeze-fracture studies

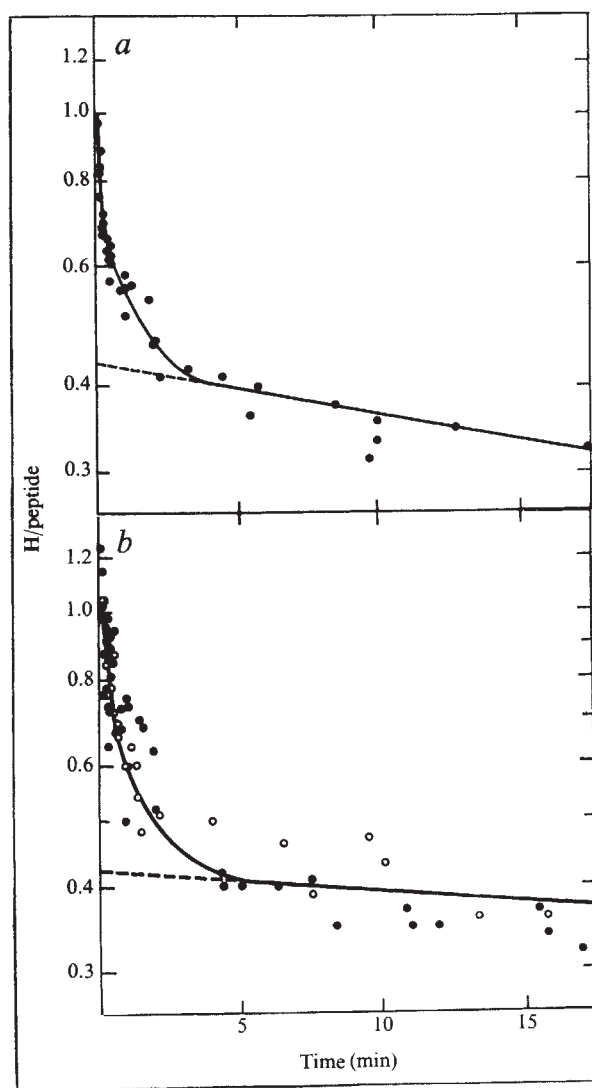
of native photoreceptor membranes<sup>28</sup>. Also against such a model is the consideration that this placement of the polypeptide chain seems to have little potential for function.

A more compelling though still quite general possibility is that a good portion, perhaps 50%, of the polypeptide chain is arranged so that it forms the surface of an aqueous channel into the membrane. In order to traverse a considerable fraction of the lipid bilayer and also contain enough bulk water to satisfy the requirement for hydrogen bonding to peptides at its surface, the channel would have to be of the order of 10–15 Å in diameter. Analogous arrangements may be considered if two or more monomers cooperate to enclose the aqueous space<sup>28</sup>.

The changes in hydrogen exchange behaviour found on illumination involve the slowly exchanging, internally bonded hydrogens corresponding to structured regions of polypeptide. The free peptide region of the curve is unaffected by light (Fig. 2). This matches the obvious expectation that the retinaldehyde chromophore is held within a structured part of the molecule, and is consistent with the supposition that this structure may act as a plug that closes off the rhodopsin channel in the dark but becomes distorted after illumination to



**Fig. 1** Hydrogen-tritium exchange-out data for disk membrane suspensions at pH 7.7, 0°C. The frog (a) and cattle (b) disk membrane preparations used had ratios  $A_{280}/A_{500}$  of 2.2–2.5 after purification on discontinuous sucrose gradients. Exchangeable hydrogen sites were labelled to equilibrium with tritium in darkness by exchanging-in for 50–60 h at 0°C in Ringer buffer with added tritiated water. Exchange-out was initiated (zero time) by passing a sample of the suspension through a Sephadex column to remove the free tritiated water. Subsequent separations allowed measurement of the amount of tritium still remaining bound as a function of exchange-out time, and the data were computed and plotted in terms of mol of original hydrogen as yet unexchanged per mol of peptide group present. a, Disk membranes from frogs were either dark-adapted (●) or irradiated with white light at 0°C for several minutes starting at zero time of the exchange-out experiment (○). When disk membranes from cattle were so treated (except that irradiation was with light of  $\lambda > 540$  nm) no changes in exchange behaviour developed relative to the dark control. b, Exchange-out of cattle disk membranes in the dark-adapted state (●) is compared with that of disk membranes containing opsin (○). Here the rhodopsin was converted to opsin by bleaching in white light and then incubating the disk membrane suspension for 45 min at 35°C. The suspensions were recooled to 0°C before beginning exchange-out. We do not know if the difference in response between cattle and frog disks represents a difference in the intermediates formed or in their kinetics of formation.



**Fig. 2** Exchange-out data for frog (a) and cattle (b) disk membranes at pH 5.3 and 0°C. Data from dark-adapted (●) and opsin-containing disks (○) are shown. In both curves, 0.4 H/peptide group are slow and 0.7 form a faster kinetic class exchanging at just the free-peptide rate.

allow the rapid escape of some transmitter substance contained within the disk membrane.

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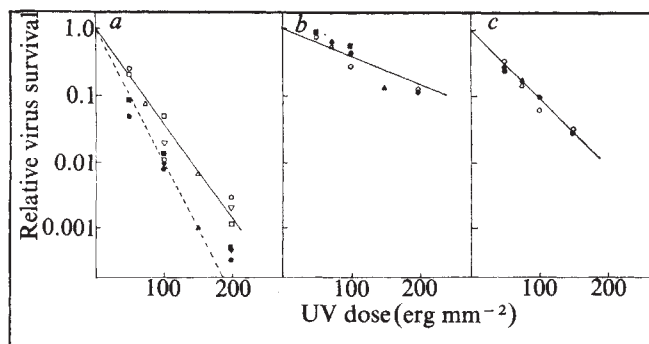
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- 1 Wald, G., Brown, P. K., and Gibbons, I. R., *J. Opt. Soc. Am.*, **53**, 20-35 (1963).
- 2 Hagins, W. A., *A. Rev. Biophys. Bioeng.*, **1**, 131-158 (1972).
- 3 Szuts, E. Z., and Cone, R. A., *Fedn Proc. Abstr.*, **33**, II, 1471 (1974).
- 4 Englander, S. W., and Englander, J. J., *Methods Enzymol.*, **26C**, 406-413 (1972).
- 5 Englander, S. W., Downer, N. D., and Teitelbaum, H., *A. Rev. Biochem.*, **41**, 903-24 (1972).
- 6 Wald, G., and Brown, P. K., *J. gen. Physiol.*, **35**, 797-821 (1952).
- 7 Bownds, D., and Wald, G., *Nature*, **205**, 254-257 (1965).
- 8 Hubbard, R., Bownds, D., and Yoshikawa, T., *Cold Spring Harb. Symp. quant. Biol.*, **32**, 301-315 (1965).
- 9 Heller, J., *Biochemistry*, **7**, 2914-2920 (1968).
- 10 Shichi, H., Lewis, M., Irreverre, E., and Stone, A., *J. biol. Chem.*, **244**, 529-536 (1969).
- 11 Cassim, J. Y., Rafferty, C. N., and McConnell, D. G., *Biophys. Soc. Abstr.*, **12**, 205a (1972).
- 12 Molday, R. S., Englander, S. W., and Kallen, R. G., *Biochemistry*, **11**, 150-158 (1972).
- 13 Englander, S. W., and Poulsen, A., *Biopolymers*, **7**, 379-393 (1969).
- 14 Englander, S. W., and Staley, R., *J. molec. Biol.*, **45**, 277-295 (1969).
- 15 Yee, R. Y., Englander, S. W., and von Hippel, P. H., *J. molec. Biol.*, **83**, 1-16 (1974).
- 16 Bleich, E. H., Galaray, R. E., Printz, M. P., and Craig, L. C., *Biochemistry*, **12**, 4950-4957 (1973).
- 17 Borggren, J. M. P. M., Daemen, F. J. M., and Bonting, S. L., *Biochim. biophys. Acta*, **202**, 374-381 (1970).
- 18 Heller, J., and Lawrence, M., *Biochemistry*, **9**, 864-869 (1970).
- 19 Robinson, W. E., Gordon-Walker, A., and Bownds, D., *Nature new Biol.*, **235**, 112-114 (1972).
- 20 DeGrip, W. J., Daemen, F. J. M., and Bonting, S. L., *Biochim. biophys. Acta*, **323**, 125-142 (1973).
- 21 Liebman, P. A., *Biophys. Soc. Abstr.*, **12**, 99a (1972).
- 22 Heitzmann, H., *Nature new Biol.*, **235**, 114 (1972).
- 23 Papermaster, D. S., and Dreyer, W. J., *Biochemistry*, **13**, 2438-2444 (1974).
- 24 Francois, C., *Biochim. biophys. Acta*, **173**, 86-93 (1969).
- 25 Wu, C., and Stryer, L., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1104-1108 (1972).
- 26 Daemen, F. J. M., van Breugel, P. J. G. M., and Bonting, S. L., *Fedn Proc. Abstr.*, **33**, II, 1575 (1974).
- 27 Saari, J. C., *Fedn Proc. Abstr.*, **33**, II, 1576 (1975).
- 28 Chen, Y. S., and Hubbell, W. L., *Expl Eye Res.*, **17**, 517-532 (1973).



**Fig. 1** Photoreactivation of HSV in Jay Tim cells: *a*, Jay Tim; *b*, HESM; *c*, XP-1, stocks of the F strain of HSV were grown and their titre was determined as before<sup>9</sup>. Samples containing between  $10^6$  and  $10^7$  plaque forming units (PFU) of HSV in 1 ml of phosphate-buffered saline containing 0.1% glucose (PBS-glucose) were irradiated for various times with a 15-W GE germicidal lamp; ultraviolet dose was measured with a Jagger metre<sup>13</sup> calibrated with a Hewlett-Packard thermopile. Immediately after irradiation the virus stocks were diluted with PBS-glucose and overlaid on fibroblasts in 25-cm<sup>2</sup> plastic tissue culture flasks at 37 °C. Xeroderma pigmentosum cell lines were obtained from the American Type Culture Collection and normal human embryonic skin and muscle fibroblasts (HESM cells) were obtained from Flow Laboratories. The growth and maintenance of these cells in Dulbecco's modified Eagle's MEM with 20% foetal calf serum has been described<sup>8</sup>. Appropriate dilutions of virus were allowed to adsorb to the cells for 60 min in PBS-glucose and then the cells were overlaid with medium 199 containing 2% foetal calf serum and 0.4% human immune serum globulin. The infected cells were either illuminated for 6 h with a 60-W yellow incandescent bulb at an average distance of 25 cm in an incubator at 37 °C or were kept in the dark by wrapping in aluminium foil. Plaques were allowed to develop for 48 h and visualised by staining the infected cells with 2% crystal violet in 20% ethanol. Virus survival was calculated from the relative plaque titres obtained by diluting unirradiated HSV and carrying out a parallel infection in illuminated or dark cells. The open symbols represent titres on photoreactivated cells and the closed symbols represent titres on cells kept in the dark. The different symbols represent different experiments. The ultraviolet-dose curves on HESM cells were carried out in parallel with those on Jay Tim cells.

## Photoreactivation of herpes simplex virus in human fibroblasts

WHEN DNA is treated with ultraviolet light (220-300 nm) cyclobutyl pyrimidine dimers are produced between adjacent pyrimidines on a strand of DNA<sup>1</sup>. These dimers are a major cause of mutations and death in simple organisms after ultraviolet-irradiation<sup>1</sup>, and they have been implicated in the induction of skin cancer in man<sup>2,3</sup>. An important tool for assessing the deleterious effects of dimers is the photoreactivation test: the photoreactivating enzyme repairs DNA by the specific and exclusive monomerisation of dimers in a light-dependent (> 300 nm) reaction<sup>4,5</sup>. If ultraviolet biological damage can be reversed by true photoenzymatic repair, then dimers have a major role in the production of that damage<sup>6</sup>. For the test to assess the role of ultraviolet-induced dimers in human carcinogenesis at least three criteria must be met: (1) human cells must possess a functional photoreactivating enzyme, (2) this enzyme must be able to monomerise dimers in DNA, and (3) the enzyme must be able to restore biological activity to ultraviolet-irradiated DNA. It has been shown that human cells meet the first two criteria<sup>7,8</sup>; we now show that human photoreactivating enzyme in fibroblasts can restore infectivity to ultraviolet-irradiated herpes simplex virus (HSV).

We exposed HSV in phosphate-buffered saline solution<sup>9</sup> to increasing doses of ultraviolet light from a low pressure mercury arc. The ultraviolet-irradiated HSV was then titred on normal fibroblasts or those from patients with xeroderma pigmentosum, a rare genetic disease characterised by development of neoplasia in skin exposed to high levels of sunlight<sup>3</sup>. It has been found that different cell lines derived from patients with

xeroderma pigmentosum vary in both their levels of photoreactivating enzyme and the excision repair pathway<sup>3,8</sup>. The ultraviolet-irradiated virus stocks were titrated on a xeroderma pigmentosum cell line lacking excision repair but possessing photoreactivating enzyme (Jay Tim) (Table 1). Infected cells were exposed to photoreactivating light for 6 h; duplicate cultures were kept in the dark and plaques were allowed to develop (Fig. 1). Figure 1*a* demonstrates that virus exposed to 150 erg mm<sup>-2</sup> of ultraviolet light and plated in the dark on Jay Tim cells showed an apparent drop in titre by a factor of 1,000; in contrast, the titre of this virus was only reduced by a factor of 150 when cells were illuminated with photoreactivating light. Since human photoreactivating enzyme has an action spectrum that extends beyond 600 nm (ref. 16), we used a 60-W yellow incandescent light as our photoreactivating source to minimise damaging wavelengths in white incandescent light. In the experiment shown in Fig. 1*b* photoreactivation of HSV could not be detected when the virus was titred on normal human fibroblasts (HESM cells) containing both excision repair and photoreactivating enzyme (Table 2). The difficulties in detecting photoreactivation in excision-proficient cells have been discussed by Harm *et al.*<sup>10</sup>. The apparent drop in HSV titre at a given ultraviolet dose was considerably greater with xeroderma pigmentosum cells compared with normal fibroblasts. This result is in agreement with previous reports on the effect of excision repair on ultraviolet-damaged HSV<sup>11,12</sup>.

If the increased light-dependent survival of the HSV in Jay Tim cells is true photoenzymatic repair, cells which lack photoreactivating enzyme should show no effect of light on virus survival. XP-1 cells contain neither appreciable excision



**Table 1** DNA repair activity in normal and xeroderma fibroblasts

| Cell line                       | Unscheduled synthesis<br>(% of normal) | Photoreactivating enzyme activity<br>(U) | (% of normal) |
|---------------------------------|----------------------------------------|------------------------------------------|---------------|
| Human embryonic skin and muscle | 100*                                   | 627†                                     | 100           |
| Jay Tim (XP12BE)                | <2*                                    | 227†                                     | 36.3          |
| XP-1 (XP1LO)                    | <2*                                    | 2.93                                     | <1            |
| Wo Mec (XP4BE)                  | 100*                                   | 58.2                                     | 10.8          |

\*See ref. 3 for a complete discussion of unscheduled synthesis.

†Photoreactivating enzyme activity was determined by the method of Sutherland and Chamberlin<sup>14</sup>; protein was determined by the Lowry method<sup>15</sup> using bovine serum albumin as a standard. Units of enzyme activity are pmol mg<sup>-1</sup> h<sup>-1</sup>.

repair capacity nor photoreactivating enzyme activity (Table 1). HSV treated with 100 erg mm<sup>-2</sup> showed a tenfold decrease in virus titre on XP-1 cells whether the cells were treated with photoreactivating light or kept in the dark (Fig. 1c). In comparison, when cells were treated with white light from a 60-W bulb for 24 h after infection, the inactivated virus showed a 50-fold decrease in apparent titre. This was presumably the result of further damage to the infected cells by inactivating radiation present in the white light.

We tested the contribution of photoprotection<sup>13</sup> to increased virus survival as follows: Jay Tim cells were illuminated with photoreactivating light for 8 h before infection and then kept in the dark for plaque development. We detected no appreciable difference in virus titre compared with unilluminated cells. At a dose of 200 erg mm<sup>-2</sup> we found the titre of the virus reduced more than 500-fold in both cases. We therefore conclude that photoprotection does not contribute to the increased survival of HSV in Jay Tim cells treated with photoreactivating light. We can also exclude any differences in the efficiency of plaque formation of HSV on xeroderma pigmentosum cell lines compared with normal human fibroblasts as a contributor to our observed results. Several experiments with both types of cells kept in the dark yielded virus titres within 10% of each other when unirradiated HSV was used.

**Table 2** Effect of HSV infection on photoreactivity enzyme activity

| Cells                           | Infected with 10 PFU per cell HSV* | Photoreactivating enzyme activity (% of line 1) |
|---------------------------------|------------------------------------|-------------------------------------------------|
| Human embryonic skin and muscle | No                                 | 100                                             |
| Human embryonic skin and muscle | Yes                                | 68.5                                            |
| Wo Mec                          | No                                 | 10                                              |
| Wo Mec                          | Yes                                | 8.15                                            |

\*Infection was carried out as described in Fig. 1. Cells were overlaid with Medium 199 containing 2% foetal calf serum and harvested at 7 h after infection.

These results show that the increased survival of HSV is caused by true photoreactivation. It was considered possible, however, that such photoreactivation could be the result of the induction of an HSV specific enzyme after infection rather than to a human enzyme. To exclude this possibility we assayed photoreactivating enzyme levels in HESM cells and in a xeroderma pigmentosum line (Wo Mec) with a low level of photoreactivating enzyme but normal levels of excision repair activity (Table 1). The data in Table 2 show that there is no increase in the photoreactivating enzyme level in HSV-infected HESM cells. Thus virus infection does not increase photoreactivating activity *per se*. In the Wo Mec cells there was no increase in enzyme activity after HSV infection (Table 2). This cell line should be an extremely sensitive indicator of appearance of any photoreactivating enzyme synthesised *de novo* provided there is normal expression of the HSV genome. We have determined the yield of infectious virus from xeroderma pigmentosum

lines. When the cells were kept in the dark we could obtain as much as 5–10 plaque forming units (PFU) of infectious virus per infected cell compared with a yield of 10–30 PFU per infected cells with HESM cells. Such results, together with the efficiency of plaque formation of HSV on xeroderma pigmentosum cell lines, demonstrate that there is normal expression of HSV gene products during the infection of these cells. We conclude, therefore that all the photoreactivation observed in the infected cells is caused by the presence of the human enzyme.

We have demonstrated that the photoreactivating enzyme in human cells repairs UV-damaged DNA and restores its biological activity. This demonstration confirms the biological significance of the human photoreactivating enzyme. It is thus valid to use the photoreactivating test in evaluating the contribution of pyrimidine dimers in UV-induced human skin cancers.

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- 1 Setlow, R. B., *Science*, **153**, 379–386 (1966).
- 2 Epstein, J. H., in *Photophysiology*, 5, (edit. by Giese, A. C.) 235–273 (Academic, New York, 1971).
- 3 Robbins, J. H., Lutzner, M. A., Festoff, B. W., and Coon, H. G., *Ann. internal Med.*, **80**, 221–248 (1974).
- 4 Rupert, C. S., *J. gen. Physiol.*, **43**, 573–595 (1962).
- 5 Setlow, J. K., and Setlow, R. B., *Nature*, **197**, 560–562 (1963).
- 6 Cook, J. S., in *Photophysiology* (edit. by Giese, A. C.), **3**, 191–233 (Academic, New York, 1971).
- 7 Sutherland, B. M., *Nature*, **248**, 109–112 (1974).
- 8 Sutherland, B. M., Rice, M., and Wagner, E. K., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 103–107 (1975).
- 9 Wagner, E., Swanson, R., and Stafford, M., *J. Virol.*, **10**, 675–682 (1972).
- 10 Harm, W., Rupert, C. S., and Harm, H., in *Molecular and cellular repair processes* (edit. by Beers, R. F., Herriott, R. M., and Tilghman, R. C.), 53–63 (Johns Hopkins University Press, Baltimore, 1971).
- 11 Rabson, A. S., Tyrrell, S. A., Legallais, F. Y., *Proc. Soc. Exp. Biol. Med.*, **132**, 802–806 (1969).
- 12 Lytle, C. D., Aaronson, S. A., and Harvey, E., *Int. J. Radiat. Biol.*, **22**, 159–165 (1972).
- 13 Jagger, J., *Introduction to Research in Ultraviolet Photobiology* (Prentice Hall, Englewood Cliffs, New Jersey, 1967).
- 14 Sutherland, B. M., and Chamberlin, M. J., *Analyt. Biochem.*, **53**, 168–176 (1973).
- 15 Lowry, O. H., Rosebrough, M. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, **193**, 265–275 (1951).

## Cyclic AMP and the production of sex pili by *E. coli* K-12 carrying derepressed sex factors

THE filamentous appendages, known as sex pili, determined by F-like and I-like sex factors in enterobacteria, have an essential function in conjugation<sup>1</sup>. But neither the exact nature of their function, nor their mode of formation is yet known. The number of sex pili ordinarily seen in cultures of *Escherichia coli* K-12 carrying derepressed F-like or I-like sex factors<sup>2</sup> (where pilus production is not limited by specific repression) seldom exceeds an average of 1–2 per bacterium. With I-like pili, however, the numbers can be increased 50-fold or more by exposing the bacteria to either antiserum reacting with the pili<sup>3</sup>, or vigorous washing<sup>4</sup>.

Liquid cultures of strains of *E. coli* K-12 producing sex pili due to a derepressed sex factor are commonly granular in appearance<sup>5,6</sup>. Therefore, when very large clumps of bacteria visible to the naked eye were seen with a particular strain, M878, carrying the derepressed I-like sex factor, *Ird16* (ref. 7), this suggested that M878 was forming exceptionally large numbers of the sex pili determined by *Ird16*. The parent of strain M878 was PP78cya *crp*<sup>8</sup> (deficient in adenyl cyclase<sup>8</sup> and cyclic AMP receptor protein<sup>9</sup>); the tentative interpretation was,

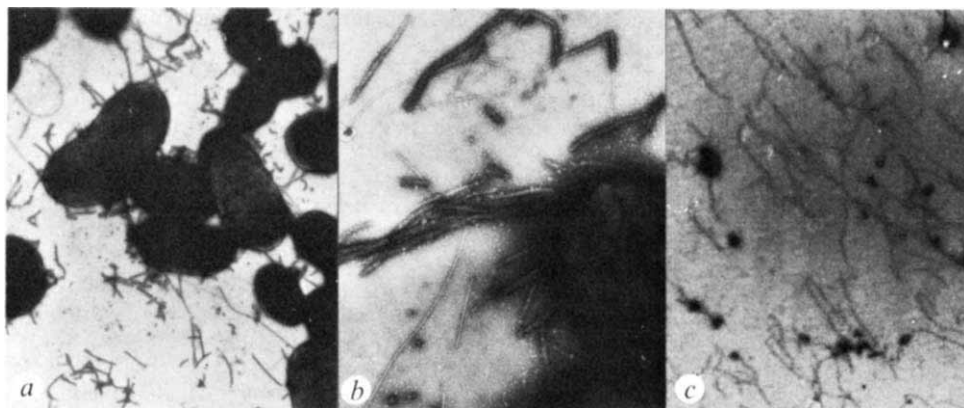


Fig. 1 *a, b*, Samples of a broth culture of strain 5336*cya crp*<sup>+</sup> carrying R538I*drd2*, grown without cyclic AMP, showing numerous I-like sex pili, distinguished from F pili and common pili by attachment of specific antibody, as described by Lawn and Meynell<sup>6</sup>. Samples were fixed with 0.5% formalin before adding antiserum to avoid the possibility of an increase in pilus production caused by the antibody itself<sup>3</sup>. Comparison with the sample in *c*, which was treated with F pilus antiserum, shows that large numbers of I-like pili were indeed also present when they had not reacted with the antibody (*a*,  $\times 7,700$ ; *b*,  $\times 23,760$ ; *c*,  $\times 16,280$ ).

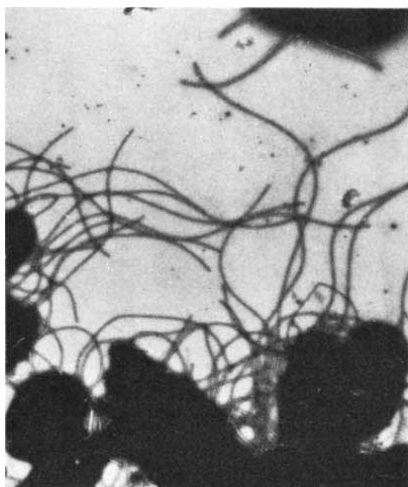
therefore, that the unusual behaviour of strain M878 was caused by the *cya crp* mutations. PP78 was derived from strain 1100 (*cya*<sup>+</sup> *crp*<sup>+</sup>) (ref. 8) which, in turn, was derived from HfrH*thi*, strain 3000 (ref. 10). In addition to carrying *ldrd16*, M878 had been made E1<sup>+</sup> (resistant to colicin E1-K30) and carried ColE1-K30 (ref. 11). The *cya*<sup>+</sup> *crp*<sup>+</sup> control strain (M889) for M878 was, therefore, strain 1100 made E1<sup>+</sup> and carrying ColE1-K30 and *ldrd16*.

When cultures of M878*cya crp* were examined by electron microscopy, at least 100-fold more I-like sex pili were seen, mainly loose and scattered over the specimen grid, compared to cultures of the control strain, M889*cya*<sup>+</sup> *crp*<sup>+</sup>. Since M878 and M889 both originated from HfrH, they were expected to form F pili, common pili and flagella, as well as I-like pili. However, only I-like pili were more numerous in M878 than M889. F pili and common pili were equally frequent in either strain but only a very rare flagellum was seen with M878. This confirms Yokota and Gots<sup>12</sup> who showed that cyclic AMP is required for flagella formation but that its absence does not decrease the numbers of F pili.

Similar results were obtained with two derepressed I-like R factors, R538I*drd2* and R64*drd11*, after their transfer to strain PP78*cya crp* E1<sup>+</sup> Col<sup>-</sup>.

To test whether increased formation of I-like sex pili resulted from the *cya* or the *crp* mutation, R538I*drd2* and R64*drd11* were transferred to the following strains: 5333*cya*<sup>+</sup> *crp*<sup>0</sup>, 5336*cya crp*<sup>+</sup> (ref. 8) (from strain 1100); CA8306*cya crp*<sup>+</sup> (from HfrH, strain 3000); and WZ25*cya*<sup>+</sup> *crp* F<sup>-</sup> (ref. 13). In each case, large numbers of I-like sex pili were formed. Thus the products of both the *cya* and the *crp* genes were involved in restraint of pilus formation and their effect was not confined to strain M878, originally tested.

Fig. 2 Sample from a broth culture of strain CA8306*cya crp*<sup>+</sup> carrying R538I*drd2*, grown with 0.33 mM cyclic AMP. Large numbers of flagella, but no I-like sex pili, can be seen ( $\times 10,000$ ).



The effect of the *cya* mutation on synthesis of catabolite-sensitive enzymes is reversed by adding cyclic AMP to the growth medium<sup>8</sup>. Strain 5336*cya* or strain CA8306*cya* carrying R538I*drd2* were grown either with or without 0.33 mM cyclic AMP. The two strains behaved identically; without cyclic AMP, the cultures showed many I-like pili but only a rare flagellum (Fig. 1*a,b,c*); with cyclic AMP, they showed few I-like pili (considerably less than one per ten bacteria) but many more flagella than were observed in *cya*<sup>+</sup> *crp*<sup>+</sup> strains whether cyclic AMP was present or not (Fig. 2). Also, the bacterial bodies differed: with cyclic AMP, they had their usual bacillary shape, but without cyclic AMP, they were almost coccoid. The numbers of F pili or common pili were not significantly affected.

In our experience, F is not typical of derepressed F-like sex factors inasmuch as it causes relatively few sex pili to be formed. The effect of cyclic AMP was, therefore, tested on four derepressed F-like R factors (two *o*<sup>+</sup>, R538F*drd1* and R124*drd2*; and two *i*<sup>-</sup>, R1*drd19* and R136*drdM1*; refs 14, 15) in strain 5336*cya crp*<sup>+</sup>. With all four R factors, about 10 times more F-like sex pili were present in cultures grown without cyclic AMP. The contrast was less striking than with I-like factors because more pili were formed when cyclic AMP was present.

We have, as yet, no clear indication of the level at which cyclic AMP acts in restraining sex pilus production. The effect of the *cya crp* mutations could be on pilus protein synthesis or its polymerisation into morphological pili. The fact that the cyclic AMP receptor protein is also required may indicate that, as with other prokaryotic functions where cyclic AMP is involved, cyclic AMP acts at the level of transcription<sup>16</sup>. It is notable that the effect of cyclic AMP and its receptor protein on pilus production is negative rather than positive. But the *cya* or *crp* mutations need not directly affect transcription of genes involved in pilus production, but may act indirectly through some more general effect on the bacterial cell envelope<sup>17</sup>. Some authors have suggested the possibility that pilus protein may be present as a constituent of the bacterial membrane when a sex factor is present (for example ref. 18). The bacteria of the *cya crp* strain, PP78, had an altered shape and, in conditions where they carried an I-like sex factor and produced large numbers of sex pili, the bacteria were lysed by low concentrations of sodium dodecyl sulphate (SDS) ( $\sim 1\%$ ) which in the case of *cya*<sup>+</sup> *crp*<sup>+</sup> bacteria<sup>9</sup> only dissolved the sex pili without affecting the viability, microscopic appearance or motility of the bacteria. Neither did the same concentrations of SDS affect *cya crp* bacteria not carrying an I-like sex factor (K. Flint, unpublished).

Washing with broth or phosphate-buffered saline increases the number of sex pili<sup>4</sup> and removes intracellular cyclic AMP<sup>19</sup>. If the effect on pilus production caused by washing was a result of removal of cyclic AMP, it might be abolished by adding cyclic AMP to the wash medium. This could not be shown, however, despite repeated attempts. Samples of the strain, W945(R538I*drd2*), with which the effect of washing was originally demonstrated, were washed with broth containing up to 3 mM cyclic AMP neutralised with NaOH, after growth in the same medium,



but the number of pili produced was similar to control preparations grown and washed with medium without cyclic AMP.

Even where a structural component has been identified in bacterial conjugation, as with the sex pili determined by F-like and I-like sex factors, a major difficulty in its study up to now has been its isolation in sufficient quantity. This difficulty should be overcome to a considerable extent by the greatly increased numbers of pili produced by *cya crp* mutant bacteria, as described here. Furthermore, several additional categories of sex factor exist for which a structural component corresponding to the sex pili of the F-like and I-like classes has not yet been demonstrated<sup>20</sup>. If such a structure were similarly produced in greater than normal amounts in *cya crp* mutant bacteria, the use of such bacteria may increase the likelihood of its identification.

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- 1 Meynell, G. G., *Bacterial Plasmids* (Macmillan, London, 1972).
- 2 Meynell, E., Meynell, G. G., and Datta, N., *Bact. Rev.*, **32**, 55-83 (1968).
- 3 Lawn, A. M., and Meynell, E., *Nature*, **235**, 441-442 (1972).
- 4 Lawn, A. M., and Meynell, E., *J. gen. Microbiol.*, **86**, 188-190 (1975).
- 5 Stocker, B. A. D., Smith, S. M., and Ozeki, H., *J. gen. Microbiol.*, **30**, 201-221 (1963).
- 6 Lawn, A. M., and Meynell, E., *J. Hyg., Camb.*, **68**, 683-694 (1970).
- 7 Hardy, K. G., Meynell, G. G., Dowman, J. E., and Spratt, B. G., *Molec. gen. Genet.*, **125**, 217-230 (1973).
- 8 Perlman, R. L., and Pastan, I., *Biochem. biophys. Res. Commun.*, **37**, 151-157 (1969).
- 9 Emmer, M., de Crombrughe, B., Pastan, I., and Perlman, R., *Proc. natn. Acad. Sci. U.S.A.*, **66**, 480-487 (1970).
- 10 Fox, C. F., and Wilson, G., *Proc. natn. Acad. Sci. U.S.A.*, **59**, 988-995 (1968).
- 11 Harwood, C. R., and Meynell, G. G., *Biochem. biophys. Res. Commun.*, **63**, 535-540 (1975).
- 12 Yokota, T., and Gots, J. S., *J. Bact.*, **103**, 513-516 (1970).
- 13 Silverstone, A. E., Goman, M., and Scaife, J. G., *Molec. gen. Genet.*, **118**, 223-234 (1972).
- 14 Frydman, A., and Meynell, E., *Genet. Res., Camb.*, **14**, 315-332 (1969).
- 15 Meynell, E., and Cooke, M., *Genet. Res., Camb.*, **14**, 309-313 (1969).
- 16 Rickenberg, H. V., *A. Rev. Microbiol.*, **28**, 353-369 (1974).
- 17 Broman, R. L., Dobrogosz, W. J., and White, D. C., *Archs. Biochem. Biophys.*, **162**, 595-601 (1974).
- 18 Brinton, C. C., *Crit. Rev. Microbiol.*, **1**, 105-160 (1971).
- 19 Makman, R. S., and Sutherland, E. W., *J. biol. Chem.*, **240**, 1309-1314 (1965).
- 20 Brodt, P., Leggett, F., and Iyer, R., *Nature*, **249**, 856-858 (1974).

## Synthesis of ribosomal RNA during sporulation in *Bacillus subtilis*

SPORULATION in *Bacillus subtilis* is associated with specific changes in RNA metabolism required for the expression of sporulation genes. Although net RNA synthesis stops abruptly at the end of logarithmic growth ( $T_0$ ), active turnover and differential transcription of the genome have been demonstrated<sup>1-3</sup>. Losick and Sonnenshein<sup>4</sup> suggested that modifications of RNA polymerase may play a primary role in the regulation of gene expression during the sporulation process. The expression of ribosomal RNA genes during this period has been subject to conflicting reports<sup>1,5-7</sup>. The reported turn-off of these genes at the onset of sporulation<sup>5,6</sup> has not been observed in similar experiments<sup>7</sup>. The latter, and the reported changes in template specificity of RNA polymerase<sup>4,8</sup> were shown to be strongly dependent on the nature of the sporulation medium and to a lesser extent on the phenotype ( $sp^+$  or  $sp^-$ ) of the strain<sup>7,8</sup>. We have analysed the pattern of synthesis and turnover of stable RNA components in *B. subtilis* induced to sporulate in the highly defined resuspension medium, SM (ref. 9). We report that rRNA synthesis does continue during sporulation, although at a reduced rate, resulting in the gradual replacement of vegetative rRNA components by similar newly synthesised sporulation species.

To label the stable RNA components during vegetative growth, *B. subtilis* strain A26, a uracil auxotroph, was cultured

in a synthetic medium containing <sup>3</sup>H-uridine. These cells were centrifuged, resuspended in SM medium and samples were withdrawn at various times for a second labelling with <sup>32</sup>P-phosphate. Figure 1 illustrates the periods of labelling and some typical events connected with sporulation which were examined here, that is, nucleic acid synthesis, formation of alkaline phosphatase and heat-resistant cells. The appearance of these events are characteristic of cells sporulating in SM medium<sup>9</sup>. RNAs were extracted<sup>14</sup> in the presence of 2 mg ml<sup>-1</sup> bentonite<sup>15</sup> with 1% sodium lauryl sulphate added to protect the lysates from the new sporulation nucleases<sup>16,17</sup>. To evaluate the distribution of the labels among the RNA species and to eliminate the extraneous <sup>32</sup>P-labelled material known for its nonspecific binding to nitrocellulose membranes, the RNA preparations were first fractionated on methylated albumin-Kieselguhr (MAK) columns<sup>18</sup>. Figure 2 illustrates a typical MAK profile of an RNA sample taken from stage  $T_2$  and cochromatographed with vegetative marker. The bulk of the <sup>3</sup>H-labelled species chromatographed as stable RNA components with the expected 2:1 ratio of label in the 23S and 16S peaks, whereas the <sup>32</sup>P-labelled species had a ratio nearer to 1:1 indicating the presence of mRNA in addition to the stable RNAs. The profile also shows the efficient separation of the nonspecific <sup>32</sup>P-labelled material and low molecular weight species from the bulk RNA which eluted only after the application of the salt gradient. We calculated that in the RNAs from stages  $T_1$ ,  $T_{2.5}$ ,  $T_5$ , 15%, 30% and 60% of the <sup>3</sup>H-label eluted as low molecular weight RNA, respectively. This change

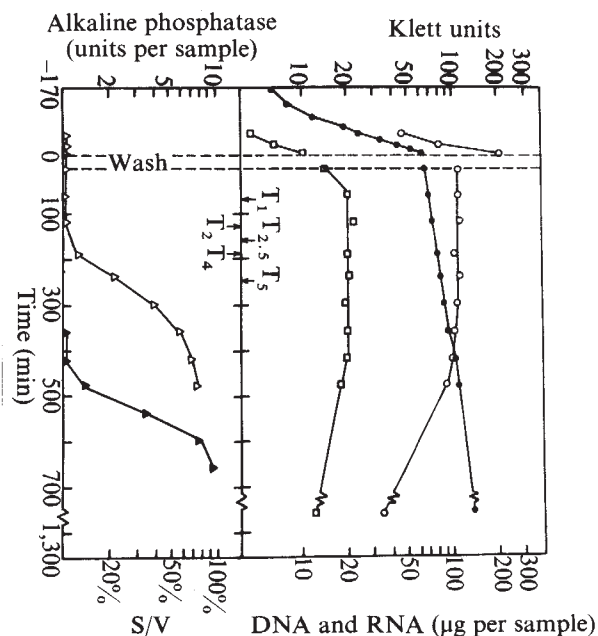


Fig. 1 Sporulation of *B. subtilis* in SM resuspension medium. A uracil auxotroph strain (A26u,  $sp^+$ ) was grown in the presence of 5,6-<sup>3</sup>H-uridine (10  $\mu$ Ci ml<sup>-1</sup>; specific activity 46.2 Ci mmol<sup>-1</sup>, New England Nuclear) and 15  $\mu$ g ml<sup>-1</sup> of unlabelled uridine. Turbidity ( $\bullet$ ) during growth was monitored by a Klett colorimeter using a 66 filter. At a Klett of 44, 100  $\mu$ g ml<sup>-1</sup> of unlabelled uridine was added for a chase period of 20 min, the cells then were resuspended in SM medium containing 15  $\mu$ g uridine ml<sup>-1</sup> for 20 h. Throughout the experiment, duplicate samples (9.5 ml) were removed and precipitated with cold TCA (final concentration 5%) and fractionated according to the Schmidt-Thannhauser procedure<sup>10</sup>. RNA ( $\square$ ) was determined by the orcinol reaction<sup>11</sup>; and DNA ( $\circ$ ) by the diphenylamine reaction<sup>12</sup>. For the estimation of alkaline phosphatase activity ( $\Delta$ ), 5 ml samples were removed and treated according to Torriani<sup>13</sup>. Spores were estimated by viable counts before (V) and after heating (S) for 10 min at 80 °C and is expressed as sporulation frequency ( $\blacktriangle$ , S/V  $\times$  100). At the indicated times after resuspension ( $T_1$ ,  $T_2$ ,  $T_{2.5}$ ,  $T_4$ , and  $T_5$ ), 200 ml samples were removed and labelled with <sup>32</sup>P-phosphate (33-70  $\mu$ Ci ml<sup>-1</sup>) for 30 min followed by rapid chilling with an equal volume of frozen media.

**Table 1** Hybridisation-competition behaviour of vegetative rRNA of *B. subtilis*

| Expt no. | DNA Preparation no. | Strand | DNA/RNA input ratio | Unlabelled rRNA competitor* | Complexed after RNase (c.p.m.)† | <sup>3</sup> H-RNA hybridised (%) | DNA (H) or (L) hybridised (%) |
|----------|---------------------|--------|---------------------|-----------------------------|---------------------------------|-----------------------------------|-------------------------------|
| 1        | I                   | H      | 50:1                | —                           | 1,650                           | 36.6                              | 0.73                          |
|          |                     | H      | 50:1                | 50 × (V)                    | 18                              | 0.4                               |                               |
| 2        | I                   | H      | 75:1                | —                           | 2,215                           | 49.1                              | 0.67                          |
|          |                     | H      | 75:1                | 50 × (V)                    | 45                              | 1.0                               |                               |
| 3        | II                  | H      | 200:1               | —                           | 4,490                           | 99.7                              | 0.50                          |
|          |                     | H      | 200:1               | 100 × (V)                   | 43                              | 1.0                               |                               |
|          |                     | H      | 200:1               | 100 × (S)                   | 167                             | 3.7                               |                               |
| 4        | II                  | L      | 95:1                | —                           | 2                               | 0.04                              | 0.0005                        |

\* Unlabelled messenger-free rRNA from vegetative (V) cells was extracted after treatment with rifampicin (30 µg ml<sup>-1</sup>) for 20 min. Similarly, unlabelled RNA from sporulating (S) cells at stage T<sub>2.5</sub> was extracted after treatment with rifampicin (100 µg ml<sup>-1</sup>) for 40 min.

† Each reaction mixture contained 0.17 µg <sup>3</sup>H-rRNA (specific activity, 26,500 c.p.m. µg<sup>-1</sup>) MAK-fractionated H or L strands, 6 × SSC in a total volume of 1.60, 1.28, 0.68 and 3.0 ml for experiments 1, 2, 3 and 4, respectively. The reactions were incubated at 67 °C for 18 h and treated as described previously<sup>18,19</sup>.

represented a turnover rate during sporulation of 12% h<sup>-1</sup>. Similar calculations were not carried out with the <sup>32</sup>P-labelled RNAs because of the high content of extraneous phosphate.

We have previously shown that in *B. subtilis* 16 and 23S rRNA, all soluble RNA (4 plus 5S) and 80–90% of pulse-labelled RNA synthesised by vegetative cells hybridise to the H strand of DNA<sup>18,19</sup>. Ribosomal cistrons account for only a small portion of the *B. subtilis* genome<sup>20</sup> but their transcription products constitute more than 95% of the total cellular contents<sup>20</sup>. The use of MAK-fractionated L and H strands of *B. subtilis* DNA enabled us to achieve nearly quantitative hybridisation of stable and pulse-labelled RNAs (refs 18 and 19). The hybridisation properties of MAK-fractionated H strands to vegetative <sup>3</sup>H-labelled rRNA are summarised in Table 1. The results shown confirm the following earlier observations: none of the rRNA hybridised to the L strand; maximum hybridisation (99.7% input) was achieved at DNA/RNA ratios

of 200 : 1; 0.25–0.37% *B. subtilis* DNA hybridised with rRNA; addition of excess (50–100 ×) unlabelled messenger-free rRNA from vegetative or sporulating cells competed entirely with the available DNA sites. The proportion of rRNA transcripts in the radioactive RNAs was determined by similar competition assays using the same H-strand preparations and the same unlabelled rRNAs as competitors. The results in Table 2 are presented as a comparison between two pulse-labelled log-phase RNAs (experiments 1 and 2) and two independent sets of double labelled sporulation RNAs (experiments 3 and 4). Hybridisation competition of the double-labelled RNAs showed that 97–98% of the vegetative <sup>3</sup>H-RNA that hybridised to the H strand was rRNA, whereas the <sup>32</sup>P-labelled sporulation RNAs were composed of both ribosomal and messenger species. The proportion of rRNA in the various <sup>32</sup>P-labelled RNAs was characteristic to the sporulation stage and related to the overall rate of RNA synthesis. One hour

**Table 2** Hybridisation-competition behaviour of RNA preparations labelled with <sup>3</sup>H-uridine during vegetative growth and with <sup>32</sup>P-phosphate during various sporulation stages of *Bacillus subtilis*

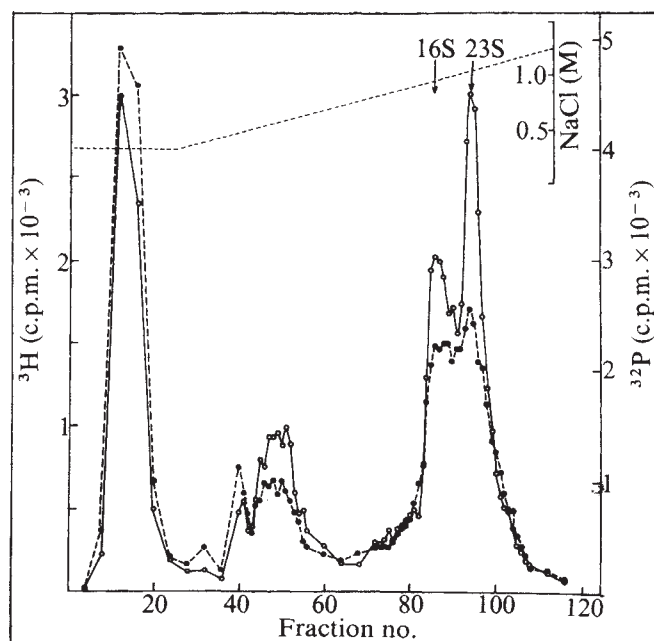
| Expt no. | Growth stage of pulse-labelling* | DNA/RNA input ratio† | Unlabelled competitor rRNA‡ | Complexed after RNase (c.p.m.) |                 | RNA hybridised (%) |                 | rRNA in labelled preparation (%) |                 |
|----------|----------------------------------|----------------------|-----------------------------|--------------------------------|-----------------|--------------------|-----------------|----------------------------------|-----------------|
|          |                                  |                      |                             | <sup>3</sup> H                 | <sup>32</sup> P | <sup>3</sup> H     | <sup>32</sup> P | <sup>3</sup> H                   | <sup>32</sup> P |
| 1        | Log (3)                          | 200 : 1              | —                           | 2,497                          | —               | 75.4               | —               | —                                | —               |
|          |                                  | 200 : 1              | 100 × (V)                   | 1,422                          | —               | 43.0               | —               | 57.0                             | —               |
|          |                                  | 200 : 1              | 100 × (S)                   | 1,425                          | —               | 43.0               | —               | 57.0                             | —               |
| 2        | Log (8)                          | 200 : 1              | —                           | 1,588                          | —               | 98.0               | —               | —                                | —               |
|          |                                  | 200 : 1              | 100 × (V)                   | 599                            | —               | 35.2               | —               | 64.1                             | —               |
|          |                                  | 200 : 1              | 100 × (S)                   | 524                            | —               | 33.0               | —               | 66.4                             | —               |
| 3        | T <sub>1</sub>                   | 75 : 1               | —                           | 20,887                         | 1,190           | 99.9               | 87.0            | —                                | —               |
|          |                                  | 75 : 1               | 50 × (V)                    | 565                            | 856             | 2.8                | 62.5            | 97.2                             | 28.2            |
|          | T <sub>2.5</sub>                 | 75 : 1               | —                           | 5,360                          | 890             | 99.9               | 55.2            | —                                | —               |
|          |                                  | 75 : 1               | 50 × (V)                    | 84                             | 325             | 1.8                | 20.2            | 98.2                             | 63.4            |
|          | T <sub>5.0</sub>                 | 75 : 1               | —                           | 8,441                          | 637             | 70.2               | 42.1            | —                                | —               |
|          |                                  | 75 : 1               | 50 × (V)                    | 109                            | 183             | 0.9                | 12.1            | 98.3                             | 72.3            |
| 4        | T <sub>2.0</sub>                 | 200 : 1              | —                           | 2,546                          | 1,006           | 99.9               | 93.1            | —                                | —               |
|          |                                  | 200 : 1              | 100 × (V)                   | 56                             | 337             | 2.2                | 31.2            | 97.8                             | 66.5            |
|          |                                  | 200 : 1              | 100 × (S)                   | 43                             | 341             | 1.7                | 31.5            | 98.3                             | 66.2            |
|          | T <sub>4.0</sub>                 | 200 : 1              | —                           | 2,497                          | 1,742           | 98.7               | 68.2            | —                                | —               |
|          |                                  | 200 : 1              | 100 × (V)                   | 50                             | 407             | 2.2                | 15.9            | 97.8                             | 76.7            |
|          |                                  | 200 : 1              | 100 × (S)                   | 40                             | 377             | 1.9                | 14.8            | 98.1                             | 78.3            |
|          |                                  | 200 : 1              | —                           | 2,497                          | 1,742           | 98.7               | 68.2            | —                                | —               |
|          |                                  | 200 : 1              | 100 × (V)                   | 50                             | 407             | 2.2                | 15.9            | 97.8                             | 76.7            |
|          |                                  | 200 : 1              | 100 × (S)                   | 40                             | 377             | 1.9                | 14.8            | 98.1                             | 78.3            |

\* Log (3) and Log (8) are log-phase cells grown in minimal salts<sup>28</sup> or in SM growth medium and pulse-labelled with <sup>3</sup>H-uridine (44 or 17 µCi ml<sup>-1</sup>) for a period of 3 and 8 min, respectively. In experiments 3 and 4 the same amount of <sup>3</sup>H-uridine (10 µCi ml<sup>-1</sup>) was used, and the amount of <sup>32</sup>P-phosphate as µCi ml<sup>-1</sup> was 33 for T<sub>1</sub>, T<sub>2.5</sub> and T<sub>5.0</sub>, 65 for T<sub>2</sub> and 70 for T<sub>4</sub>.

† The same H strand preparations described in Table 1 were used here; preparation I in experiment 3 and preparation II in 1, 2, and 4.

‡ The various reaction mixtures contained the following amounts of RNA in µg: experiments 1 and 2, 0.06 and 0.35 (specific activities (c.p.m. µg<sup>-1</sup>), 55, 200 and 4,630, respectively); experiment 3, 2.87 (specific activity (c.p.m. µg<sup>-1</sup>) of <sup>3</sup>H and <sup>32</sup>P, 7, 270 and 476) 0.83 (6,500 and 1,955) and 1.68 (7,160 and 900) for T<sub>1</sub>, T<sub>2.5</sub> and T<sub>5.0</sub>, respectively; experiment 4, 0.5 (5,130 and 2,162) 0.5 (4,400 and 5,110) for T<sub>2</sub> and T<sub>4</sub> respectively. The reaction mixture contained the appropriate amounts of MAK-fractionated H strand, 6 × SSC in a total volume of 0.68 for experiments 1, 2, and 4 and 3.0 ml for experiment 3. The reactions were incubated at 67 °C for 18 h and treated as described previously<sup>18,19</sup>.





**Fig. 2** Chromatography of double-labelled RNA  $^3\text{H}$  ( $\circ$ );  $^{32}\text{P}$  ( $\bullet$ ) from sporulation stage  $T_{2.0}$  on a MAK column. Isolated labelled RNA (0.1 mg, specific activity as c.p.m.  $\mu\text{g}^{-1}$  of 5,130 and 2,160 for  $^3\text{H}$  and  $^{32}\text{P}$ , respectively) mixed with unlabelled marker RNA (1.06 mg) from vegetative cells, was applied to a MAK column ( $1.9 \times 8.0$  cm) and eluted using a linear salt gradient between 0.3 and 1.4 M NaCl in 50mM sodium phosphate, pH 6.7 (total volume, 600 ml). Fractions (4 ml) were collected and 1.0 ml samples were mixed with 3.0 ml scintillation mixture<sup>27</sup>. The arrows indicate the peak absorbance ( $A_{260}$ ) of the unlabelled 16S and 23S marker RNA. Recovery of the unlabelled RNA (% input): total, 90.7; peak 1 (fractions 1–24), 9.2; 4S + 5S RNA (fractions 36–60), 14.3; and 16S + 23S RNA (fractions 72–105), 67.2.

after resuspension in the sporulation medium ( $T_1$ ), only 28% of the  $^{32}\text{P}$ -labelled RNA that hybridised to the H strand contained rRNA sequences, whereas at later stages,  $T_{2-2.5}$  and  $T_{4-5}$ , 66% and 78% of the  $^{32}\text{P}$  label consisted of rRNA. The latter values are comparable to the fraction of rRNA measured in pulse-label RNAs from vegetative bacteria (see experiments 1 and 2, Table 2).

We interpret the initial reduction in the labelling of rRNA at stage  $T_1$  as being comparable to the temporary retardation of RNA synthesis and accumulation observed during a carbon-source step-down or amino acid deprivation of both relaxed and stringent bacteria<sup>22,23</sup>. The SM resuspension medium<sup>9</sup> used here represents such a typical shift-down condition, which resulted in the characteristic decrease in the rate of RNA synthesis and more significantly in an RNA preparation that exhibited a smaller proportion of the label in rRNA. As soon as the cells adjusted to the resuspension medium, the rate of RNA synthesis, particularly that of rRNA, increased. When the pattern of RNA synthesis is studied in two commonly used sporulation media, the modified Schaeffer medium<sup>24,25</sup>, or the Sonenshein and Roscoe medium<sup>26</sup> containing high levels of amino acids and glucose, this initial decrease in the rate of RNA synthesis was not observed<sup>3</sup>. Instead, incorporation of radioactive precursors into RNA during 1-min pulse periods revealed a reproducible pattern of three periods of high rates of RNA synthesis at stages 0 to I, III and IV, the latter two were also observed in the SM medium used here (ref. 3 and D. T. and R. R., unpublished). We conclude that during the periods of increased RNA synthesis in sporulating *B. subtilis* a substantial portion (65–80%) of the transcripts are copies of stable RNA genes. The initial decrease in the rate and extent of rRNA synthesis which occurs only in certain media is not unique to sporulation, but rather represents the response of cells to growth rate transitions or to their entry into stationary phase. This response involves the preferential restriction of rRNA transcrip-

tion until the cells become committed to sporulation or simply cease growing and remain stationary until lysis ensues. We are currently investigating the transcription of rRNA cistrons during stationary phase of asporogeneous mutants of *B. subtilis*.

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**Note added in proof:** Pero *et al.* (*Spore*, VI (in the press)) report that in *B. subtilis* sporulating in 121B medium<sup>26</sup> only 10% of a 3-min pulse-labelled RNA ( $T_2$ ) represented ribosomal RNA.

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- <sup>1</sup> Blassa, G., *Ann. Inst. Pasteur Paris*, **110**, 175 (1966).
- <sup>2</sup> DiCioccio, R. A., and Strauss, N., *J. molec. Biol.*, **77**, 325 (1973).
- <sup>3</sup> Sumida-Yasumoto, C., and Doi, R. H., *J. Bact.*, **117**, 775 (1974).
- <sup>4</sup> Losick, R., and Sonenshein, A. L., *Nature*, **224**, 35 (1969).
- <sup>5</sup> Hussey, C., Losick, R., and Sonenshein, A. L., *J. molec. Biol.*, **57**, 59 (1971).
- <sup>6</sup> Hussey, C., Pero, J., Shorenstein, R. G., and Losick, R., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 407 (1972).
- <sup>7</sup> Bonamy, C., Hirschbein, L., and Szulmajster, J., *J. Bact.*, **113**, 1296 (1973).
- <sup>8</sup> Murray, C. D., Pun, P., and Strauss, N., *Biochem. biophys. Res. Commun.*, **60**, 295 (1974).
- <sup>9</sup> Sterlini, J. M., and Mandelstam, J., *Biochem. J.*, **113**, 29 (1969).
- <sup>10</sup> Schmidt, G., and Thannhauser, S. J., *J. biol. Chem.*, **161**, 83 (1945).
- <sup>11</sup> Schneider, W. C., *Methods in Enzymology*, 3 (edit. by Colowick, S. P., and Kaplan, N. O.), 680 (Academic, New York, 1957).
- <sup>12</sup> Burton, K., *Methods in Enzymology*, **12**, part B (edit. by Grossman, L., and Moldave, K.), 163 (Academic, New York, 1968).
- <sup>13</sup> Torriani, A., *Biochim. biophys. Acta*, **38**, 460 (1960).
- <sup>14</sup> Oishi, M., and Sueoka, N., *Proc. natn. Acad. Sci. U.S.A.*, **54**, 483 (1965).
- <sup>15</sup> Fraenkel-Conrat, H., Singer, B., and Tsugita, A., *Virology*, **14**, 54 (1961).
- <sup>16</sup> Kerjan, P., and Szulmajster, J., *Biochem. biophys. Res. Commun.*, **59**, 1079 (1974).
- <sup>17</sup> Kanamori, N., Sakabe, K., and Okazaki, R., *Biochim. biophys. Acta*, **335**, 155 (1973).
- <sup>18</sup> Margulies, L., Remeza, V., and Rudner, R., *J. Bact.*, **103**, 560 (1970).
- <sup>19</sup> Margulies, L., Remeza, V., and Rudner, R., *J. Bact.*, **107**, 610 (1971).
- <sup>20</sup> Smith, I., Dubnau, D., Norell, P., and Marmur, J., *J. molec. Biol.*, **33**, 123 (1968).
- <sup>21</sup> Midgley, J. E. M., *Biochem. J.*, **115**, 171 (1969).
- <sup>22</sup> Maaloe, O., and Kjeldgaard, N. O., *Control of Macromolecular Synthesis*, 97 (Benjamin, New York and Amsterdam, 1966).
- <sup>23</sup> Lazzarini, R. A., and Winslow, R. M., *Cold Spring Harb. Symp. quant. Biol.*, **35**, 383 (1970).
- <sup>24</sup> Schaeffer, P., Millet, J., and Aubert, J. P., *Proc. natn. Acad. Sci. U.S.A.*, **54**, 571 (1965).
- <sup>25</sup> Leighton, T. J., and Doi, R. H., *J. biol. Chem.*, **246**, 3189 (1971).
- <sup>26</sup> Sonenshein, A. L., and Roscoe, D. H., *Virology*, **39**, 265 (1969).
- <sup>27</sup> Bray, G. A., *Analyt. Biochem.*, **1**, 279 (1970).
- <sup>28</sup> Spizizen, J., *Proc. natn. Acad. Sci. U.S.A.*, **44**, 1072 (1958).

## Mefloquine, a clinically useful quinolinemethanol antimalarial which does not significantly bind to DNA

We report that mefloquine (2,8-bis-(trifluoromethyl)- $\alpha$ -(2-piperidyl)-4-quinolinemethanol<sup>1</sup>, see Fig. 1) clinically useful against drug-resistant *Plasmodium falciparum* malaria (G. M. Trenholme, unpublished), does not significantly bind to calf thymus DNA. This constitutes the first reported case of an active compound in the quinoline-acridine class of antimalarials which does not strongly bind to DNA by intercalation.

Investigations of the binding of acridine derivatives to DNA have provided information both on DNA structure and on the manner in which the complex of the dye with DNA can affect physiological functions. Lerma<sup>2</sup>, for example, tested the intercalation theory, using quinacrine, and Hahn<sup>3</sup> has carried out detailed spectroscopic investigations on the interaction of this compound with DNA. An entire group of antimalarial compounds has been termed the quinoline-acridine class by Pinder<sup>4</sup>. These compounds consist of a planar aromatic ring system with a positively charged side chain attached, and are typified by the quinolines, chloroquine and quinine, and the acridine, quina- crine. Considerable evidence has accumulated that these three antimalarial compounds form an intercalated complex with DNA *in vitro* and can inhibit bacterial DNA and RNA synthesis by formation of an *in vivo* complex with DNA (ref. 5). This has led to a theory that the antimalarial action of these compounds results from such a complex in the malarial parasite<sup>5</sup>. Some controversy over this theory has arisen<sup>6-8</sup> but alternative postulations for the mode of action of these compounds are

somewhat qualitative at present. Recent synthetic efforts to find compounds which have good activity against chloroquine-resistant strains of malaria have resulted in a large number of compounds which can be used to study the postulated modes of drug action<sup>9,10</sup>. As part of a continuing investigation of DNA interaction with antimalarials of the quinoline-acridine type, we initiated a DNA binding study of some new compounds which may be considered members of this class<sup>11,12</sup>. This report extends our studies to include quinoline methanols.

One method for determining whether an aromatic compound forms a complex with DNA is to follow shifts induced in its electronic absorption spectrum on the addition of DNA. Figure 2 shows the ultraviolet spectra of mefloquine and quinine in the presence and absence of DNA. The spectrum of quinine shows the familiar hypochromism and a shift of absorption maxima to longer wavelengths typical of compounds that bind to DNA, whereas that of mefloquine undergoes no significant change. Thermal denaturation profiles conducted at a DNA phosphate and mefloquine concentration of  $10^{-4}$  M changed DNA  $T_m$  by less than one degree. The lack of significant change in  $T_m$  at this rather high ratio of compound to DNA phosphate also indicates at most a very weak interaction between mefloquine and DNA.

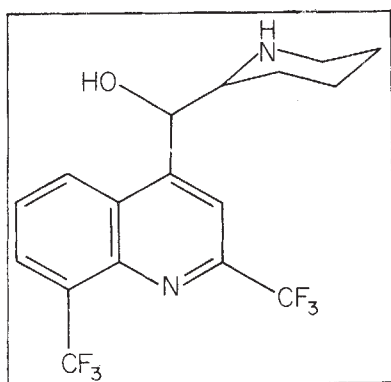


Fig. 1 Structure of mefloquine. The sample used in these studies gave NMR, infrared, ultraviolet and melting point data in accord with published values.

To quantify the mefloquine interaction with DNA, equilibrium dialysis studies were conducted using the buffer described in Fig. 2,  $2 \times 10^{-4}$  M DNA phosphate, and mefloquine concentrations from  $3.5 \times 10^{-5}$  to  $7.5 \times 10^{-5}$  M (final concentration of unbound mefloquine after equilibration). The amount of mefloquine bound varied from approximately 3.5% to 6% of the total amount added and was too low for a Scatchard binding analysis. Some estimation of the possible range of the binding constant is possible, however, if the number of mefloquine molecules bound per DNA phosphate is arbitrarily fixed. For example, if one molecule binds for every DNA phosphate (electroneutrality) then the equilibrium constant can range from 400 to 700. If one molecule binds to every five nucleotides (mefloquine/DNA phosphate = 0.2), then the binding constant must be between 2,000 and 3,700. This is quite small compared with the range of  $10^5$  to  $10^7$  typically obtained for the binding equilibrium constant of drugs, the mode of action of which is known to involve binding to DNA. A more detailed equilibrium dialysis study is impossible because of the limitations imposed on DNA concentration by the Donnan effect (at low ionic strength) and on the mefloquine concentration by the limitations of spectrophotometric determination of concentration.

Almost any positively charged compound will become associated with DNA in a weak electrostatic complex as the ionic strength is reduced to low values. To determine whether the weak binding observed with mefloquine is such an external electrostatic association or an intercalated complex, viscometric titrations have been carried out using methods described previously<sup>12</sup>. The titration curves for two intercalating com-

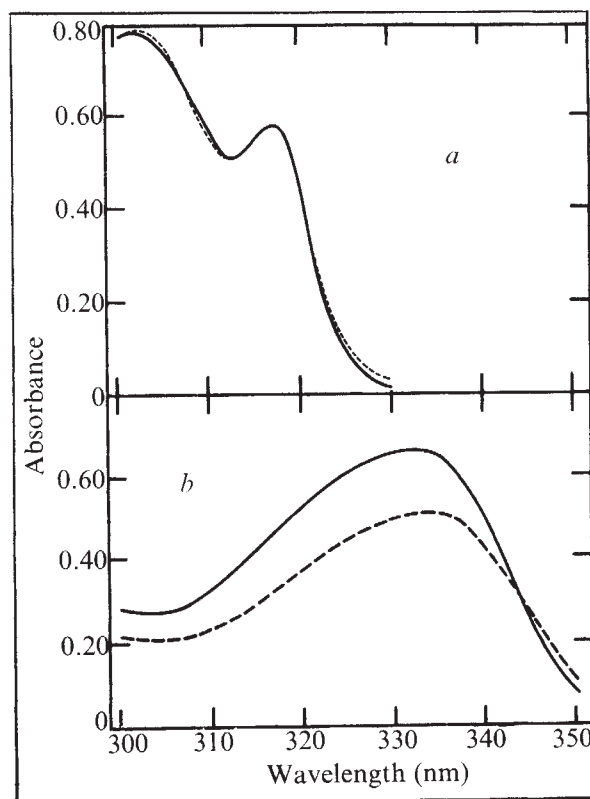
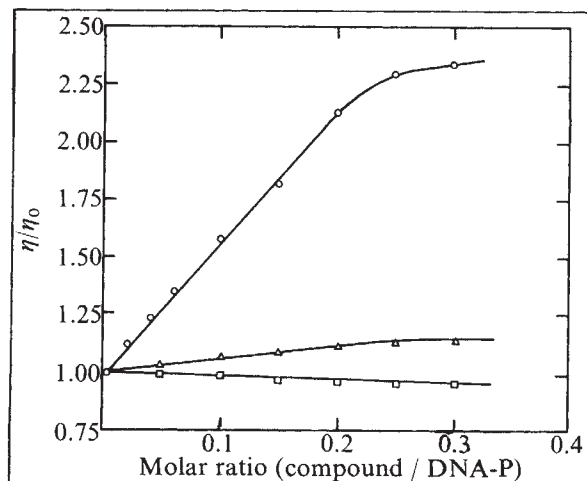


Fig. 2 Effect of DNA on the absorption spectra of mefloquine and quinine. *a*, Mefloquine hydrochloride  $2 \times 10^{-4}$  M (—); mefloquine hydrochloride  $2 \times 10^{-4}$  M plus DNA  $10^{-3}$  M (---); *b*, quinine hydrochloride  $2 \times 10^{-4}$  M (—); quinine hydrochloride  $2 \times 10^{-4}$  M plus DNA  $10^{-3}$  M (---). Solutions were prepared in  $7.5 \times 10^{-3}$  M  $\text{NaH}_2\text{PO}_4$  and  $10^{-3}$  M EDTA (standard buffer) adjusted to pH 7 with NaOH to yield an ionic strength of 0.019. Calf thymus DNA (Miles, Lot 36-155), which was used in spectrophotometric, viscometric, and thermal denaturation experiments, was dissolved in standard buffer and an extinction coefficient of 6,600/mol DNA phosphate (at 260 nm) used to calculate DNA concentration. Drug stock solutions were prepared in standard buffer and diluted to the desired concentration before absorption measurements. Difference spectra were recorded in 1-cm lightpath quartz cuvettes on a Beckman Acta V spectrophotometer. We have found that mefloquine obeys Beer's law over a concentration range considerably larger than used in these studies, suggesting that aggregation of the drug is not a problem in these conditions and cannot account for the lack of intercalation with calf thymus DNA.

pounds, a naphthothiophene methanol<sup>12</sup> and quinine, the structures of which are related to mefloquine, are shown in Fig. 3 together with the result for mefloquine. Both the compounds which intercalate cause a definite enhancement of DNA viscosity in contrast to mefloquine which causes a modest decrease in viscosity. The latter result indicates that mefloquine does not intercalate, but could bind weakly to the DNA phosphate groups through an electrostatic interaction in a manner analogous to aliphatic amines and diamines<sup>2,12</sup>.

The above results, taken together, strongly indicate that mefloquine does not intercalate with DNA, and when binding does occur at low ionic strength, it is by some weak external mechanism probably governed primarily by electrostatic interaction. A compound with the same side chain and ring system (6,8-dichloro-2-phenyl- $\alpha$ -(2-piperidyl)-4-quinolinemethanol) without the trifluoromethyl moieties but with a 2-phenyl group, has been shown to bind strongly to DNA (ref. 13). It is known that bulky groups, including trifluoromethyl groups, on aromatic ring systems can hinder intercalation<sup>14,15</sup>. Model building experiments with CPK space-filling models indicate that a 2-phenyl substituent can lie in the same plane as the quinoline ring and that the entire aromatic system can intercalate between





**Fig. 3** Viscometric titration of DNA with mefloquine hydrochloride ( $\square$ ), and quinine hydrochloride ( $\Delta$ ). The results for 6,8-dichloro-4-(2-N-piperidino-1-hydroxyethyl)naphtho[2,1-*b*]thiophene ( $\circ$ ) were taken from reference 12, in which a more detailed procedure is given. The ratio of the specific viscosity ( $\eta/\eta_0$ ) of DNA with added compound ( $\eta$ ) to DNA alone ( $\eta_0$ ) is plotted as a function of the molar ratio of compound to DNA phosphate. Viscosities were determined in a 1:2 dilution of the standard buffer with a Cannon-Ubbelohde four bulb dilution viscometer at 25 °C. A measured volume of DNA ( $2 \times 10^{-4}$  M) was successively titrated with drug stock solutions in a calibrated ( $\mu$ l) syringe, and viscosities were corrected for slight changes in DNA concentration during titration.

adjacent DNA pairs. Similar experiments with mefloquine, however, suggest that the two trifluoromethyl groups introduce unfavourable steric interactions with DNA bases in an intercalative-type complex. Since the binding constants of the smaller quinoline compounds, such as chloroquine<sup>16</sup>, are lower than similar larger aromatic compounds, such as quinacrine<sup>3</sup>, the unfavourable steric repulsion caused by the trifluoromethyl groups of mefloquine probably overrides the favourable hydrophobic interactions that would be obtained by intercalation. Both model building studies and the experimental results described above are consistent with the conclusion that mefloquine cannot bind to DNA by intercalation.

This work does not provide direct evidence for the mode of antimalarial action of mefloquine; however, it does indicate that DNA intercalation is not of primary importance and raises important questions about the mode of action of other unstudied compounds in the quinoline-acridine class of antimalarials. Identification of cellular receptors other than DNA will be important in understanding the mode of action of mefloquine and perhaps other antimalarials of this class. Since synthetic chemists have frequently assumed an intercalative DNA-binding mechanism of action similar to that of chloroquine to design potential antimalarials<sup>17-20</sup>, the fact that mefloquine binds very weakly and does not intercalate with calf thymus DNA will be important in this field. We are currently studying the interaction of several other quinoline methanol antimalarials with DNA to determine whether DNA binding could be an important part of their mode of action.

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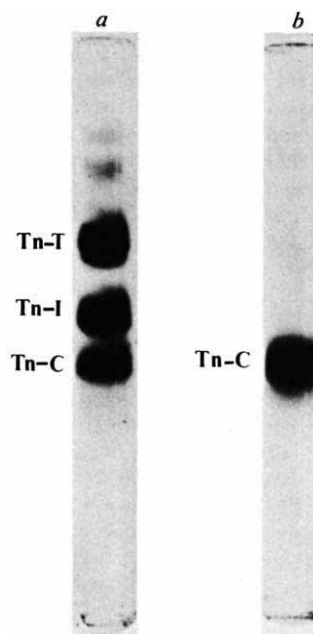
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- <sup>2</sup> Lerman, L. S., *Proc. natn. Acad. Sci. U.S.A.*, **49**, 94-102 (1963).
- <sup>3</sup> Krey, A. K., and Hahn, F. E., *Molec. Pharmac.*, **10**, 686-695 (1974).
- <sup>4</sup> Pinder, R. M., *Medicinal Chemistry* third edit., (edit. by Burger, A.), 492-521 (Wiley-Interscience, New York, 1970).
- <sup>5</sup> Hahn, F. E., O'Brien, R. L., Ciak, J., Allison, J. L., and Olenick, J. G., *Military Med.*, **131**, 1071-1089 (1966).
- <sup>6</sup> Carter, G., and Van Dyke, K., *Proc. helminth. Soc. Wash.*, **39** (special issue), 244-249 (1972).
- <sup>7</sup> Peters, W., *Postgrad. med. J.*, **49**, 573-583 (1973).
- <sup>8</sup> Howells, R. E., Peters, W., Homewood, C. A., and Warhurst, D. C., *Nature*, **228**, 625-628 (1970).
- <sup>9</sup> Warhurst, D. C., Homewood, C. A., Peters, W., and Baggaley, V. C., *Proc. helminth. Soc. Wash.*, **39** (special issue), 271-279 (1972).
- <sup>10</sup> Steck, E. A., *Chemotherapy of Protozoan Diseases*, **23**, 77-23, 177 (US Government, Washington, DC, 1972).
- <sup>11</sup> Peters, W., *Chemotherapy and Drug Resistance in Malaria*, **12** (Academic, London, 1970).
- <sup>12</sup> Panter, J. W., Boykin, D. W., and Wilson, W. D., *J. med. Chem.*, **16**, 1366-1369 (1973).
- <sup>13</sup> Olmstead, E. J., Panter, J. W., Boykin, D. W., and Wilson, W. D., *Biochemistry*, **14**, 521-526 (1975).
- <sup>14</sup> Hahn, F. E., and Fean, C. L., *Antimicrob. Ag. Chemother.*, **63**-66 (1969).
- <sup>15</sup> Muller, W., Crothers, D. M., and Waring, M. J., *Eur. J. Biochem.*, **39**, 223-234 (1973).
- <sup>16</sup> Gabbay, E. J., Glasser, R., and Gaffney, B. L., *Ann. N.Y. Acad. Sci.*, **171**, 810-825 (1970).
- <sup>17</sup> Cohen, S. N., and Yielding, K. L., *J. biol. Chem.*, **240**, 3123-3131 (1965).
- <sup>18</sup> Colwell, W. T., Brown, V., Christie, P., Lange, J., Reece, C., Yamamoto, K., and Henry, D. W., *J. med. Chem.*, **15**, 771-775 (1972).
- <sup>19</sup> Cheng, C. C., *J. Pharmaceut. Sci.*, **60**, 1596-1597 (1971).
- <sup>20</sup> Henry, D. W., *Natn. med. chem. Symp. Am. chem. Soc. Proc.*, **13**, 41-50 (1972).
- <sup>21</sup> Das, B. P., Nuss, M. E., and Boykin, D. W., *J. med. Chem.*, **17**, 516-519 (1974).

## Crystallisation of troponin-C

TROPONIN is the protein complex that controls the onset of muscular contraction<sup>1-3</sup>. It has three subunits<sup>4</sup>: troponin T (Tn-T), troponin I (Tn-I) and troponin C (Tn-C). The complex is bound at regular intervals along the thin filaments, which are composed of a double helix of actin monomers and tropomyosin, which runs along the grooves of the actin helix<sup>5-7</sup>. Each tropomyosin molecule spans seven actin monomers and one molecule of the troponin complex is bound to each tropomyosin molecule through Tn-T (refs 3 and 6-8). Tn-I binds to actin and Tn-C binds both to Tn-T and Tn-I (refs 3, 4 and 6). In the absence of Ca, Tn-I and Tn-T inhibit contraction by inhibiting the interaction of actin and myosin but in the presence of Ca, Tn-C binds to Ca, the inhibition is reversed and contraction occurs<sup>3</sup>. We have been interested in crystallising Tn-C in the hope that an eventual crystal structure determination will be helpful in understanding how Ca binding controls the onset of muscle contraction. Here we report the occurrence of a crystalline form of this protein.

**Fig. 1** SDS gel electrophoresis: *a*, of rabbit troponin (35  $\mu$ g) and *b*, Tn-C (35  $\mu$ g) separated by column chromatography (in this example, using DEAE-Sephadex A-50).



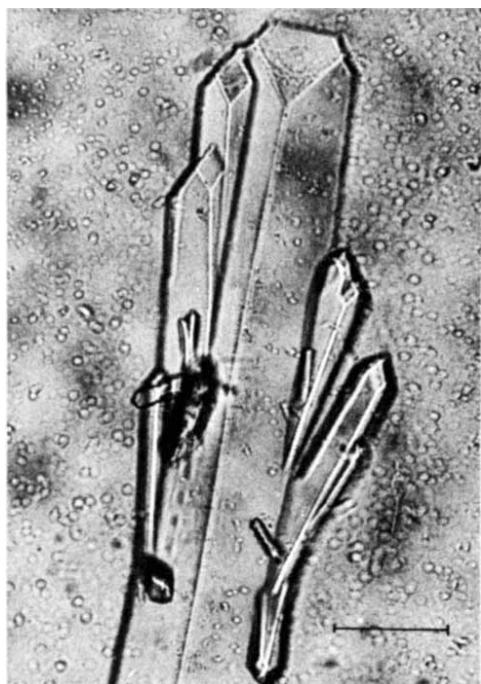


Fig. 2 Photomicrograph of crystals of pure rabbit Tn-C photographed in mother liquor with unpolarised, unfiltered light ( $\times 20$ ). Bar represents 100  $\mu\text{m}$ .

Rabbit troponin was prepared by the method of Ebashi *et al.*<sup>9</sup>. The three components were separated using column chromatography with either DEAE-Sephadex or A-50 (ref. 4) or Whatman cellulose DE-52 (ref. 6). Sodium dodecyl sulphate (SDS) gel electrophoresis<sup>10</sup> with heavy loadings of Tn-C invariably showed a single band with a molecular weight of 17–18,000 (Fig. 1) and all the Tn-C preparations, when combined with Tn-T and Tn-I, conferred Ca sensitivity on the ATPase of actomyosin.

For crystallisation a 25 mg ml<sup>-1</sup> solution of protein in 50 mM Tris-HCl 5 mM MnCl<sub>2</sub>, pH 7.0, was dialysed in Zeppenzauer cells<sup>11</sup> against 35% saturated ammonium sulphate, 5mM MnCl<sub>2</sub>, 50 mM Na acetate, adjusted to pH 5.0 with concentrated acetic acid. All chemicals were BDH reagent or AnalaR grade; no other precautions were taken. For dialysis, ordinary Visking tubing was used that had been boiled for 5 min in 0.1% NaHCO<sub>3</sub>. Crystals (Fig. 2) form within 4–8 weeks and are long bipyramidal tetragonal needles. They often show multiple growths roughly parallel to the long axis, sometimes rotated at either 45° or 90° about the long axis relative to the larger crystal.

Typically, crystals are 0.1 mm long. Occasionally large crystals (0.5  $\times$  0.05 mm) appear and these have been used for preliminary X-ray diffraction studies<sup>12</sup>. The crystals give rise to three-dimensional diffraction patterns which confirms the existence of single crystals. Rotation of the crystals by 90° about the long axis gives rise to superimposable precession photographs. The patterns are weak, however, and generally limited to 8–10 Å resolution, occasionally to 3 Å. The observed reflections may be indexed on an 'I' lattice and there is evidence (G. Dodson, B. B., D. M., and J. P., unpublished) for a 4<sub>1</sub> or 4<sub>3</sub> screw axis parallel to the long dimension of the crystals. Thus at low resolution the crystals seem to be tetragonal with a body centred unit cell of 127 Å  $\times$  156 Å. More extensive data will be necessary, however, to confirm and extend these results.

The role of MnCl<sub>2</sub> in the crystallisation procedure is obscure. It is known that certain divalent cations such as Mg can compete for at least two Ca sites<sup>13</sup>. For these sites, the Ca-binding constant of troponin is about  $5 \times 10^8$  mol<sup>-1</sup> in the absence of Mg, but about  $5 \times 10^6$  mol<sup>-1</sup> in the presence of

2 mM Mg. In general, all divalent cations with a crystal radius of 0.8–1.1 Å that can displace Ca are also capable of activating the ATPase of myosin in the presence of troponin-containing thin filaments inhibited by ethyleneglycol-bis-( $\beta$ -aminoethyl ether)*N,N'*-tetraacetic acid<sup>13</sup>. Mn is in this size range (0.8 Å) and we have confirmed the result of Konieczny and Weber (unpublished, cited in ref. 13) that Mn is active in stimulating the ATPase to the same extent as Ca. Smaller ions such as Mg, Zn, Co, and Ni are inactive in stimulating the ATPase<sup>13</sup>. These results closely parallel the ability of different ions to induce conformational changes in Tn-C similar to those produced by Ca, as judged by ultraviolet spectroscopy<sup>14</sup>.

The conformation and physical properties of Tn-C depend on the degree of saturation with Ca and these changes are evident in the properties of the troponin complex<sup>3,4,6,14,15</sup>. For example, Ca increases the affinity of Tn-C for Tn-I (refs 6 and 15). Similarly, Ca increases the affinity of Tn-C for Tn-T (ref. 6). It is likely that the Ca-dependent conformational changes in troponin Tn-C are involved in the control of the position of tropomyosin with respect to the actin helix<sup>3,8,16,17</sup>. In the presence of Ca, tropomyosin is moved to a position nearer the actin groove and actomyosin formation occurs. The Ca-dependent changes in Tn-C are likely to be the first steps in a series of structural events that constitute the control of muscular contraction. Thus a knowledge of the crystal structure is an important step in understanding this system.

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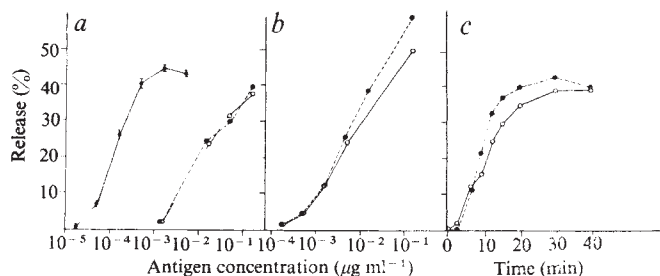
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- 1 Ebashi, S., and Kodama, A., *J. Biochem.*, **59**, 425–426 (1966).
- 2 Ebashi, S., Endo, M., and Ohtsaki, Q., *Rev. Biophys.*, **2**, 351–384 (1969).
- 3 Ebashi, S., Ohtsaki, I., and Mihashi, K., *Cold Spring Harb. Symp. quant. Biol.*, **37**, 215–223 (1972).
- 4 Greaser, M., and Gergely, J., *J. biol. Chem.*, **246**, 4226–4233 (1971).
- 5 Cohen, C., *et al.*, *Cold Spring Harb. Symp. quant. Biol.*, **37**, 287 (1972).
- 6 Margossian, S. S., and Cohen, S., *J. molec. Biol.*, **81**, 409–413 (1973).
- 7 Hanson, J., Lednev, V., O'Brien, E. J., and Bennett, P. M., *Cold Spring Harb. Symp. quant. Biol.*, **37**, 311–318 (1972).
- 8 Huxley, H. E., *Cold Spring Harb. Symp. quant. Biol.*, **37**, 361–376 (1972).
- 9 Ebashi, S., Wakabayashi, and Ebashi, F., *J. Biochem.*, **69**, 441–445 (1971).
- 10 Weber, K., and Osborn, M., *J. biol. Chem.*, **244**, 4406–4412 (1969).
- 11 Zeppenzauer, M., Ecklund, H., and Zeppenzauer, E. S., *Archs Biochem. Biophys.*, **126**, 564–568 (1968).
- 12 Potter, J. D., and Gergely, *Fedn Proc. (Abstr.)*, **33**, 1465 (1974).
- 13 Weber, A., and Murray, J. M., *Physiol. Rev.*, **53**, 612–673 (1973).
- 14 Head, J. F., and Perry, S. V., *Biochem. J.*, **137**, 145–154 (1973).
- 15 Perry, S. V., Cole, H. A., Head, J. F., and Wilson, F. J., *Cold Spring Harb. Symp. quant. Biol.*, **37**, 251 (1972).
- 16 Hazelgrove, J. C., *Cold Spring Harb. Symp. quant. Biol.*, **37**, 341–352 (1972).
- 17 Parry, D. A. D., and Squire, J. M., *J. molec. Biol.*, **75**, 33–55 (1973).

## Release of leukocyte kallikrein mediated by IgE

BRADYKININ is a vasoactive nonapeptide believed to mediate various acute inflammatory conditions<sup>1</sup>. Although its production in plasma is well defined<sup>1</sup>, an understanding of its role in disease is hampered by the lack of information about the mechanism by which it can be generated by an immune process or by any stimulus other than clotting. Immune release of a kinin-generating factor from perfused sensitised guinea pig lung has been reported<sup>2,3</sup>. The release is not, however, calcium-dependent and the factor may be a product not of the primary allergic reaction, but of a secondary event<sup>4</sup>. Substantial information is available, however, about the release of other mediators of inflammatory or allergic reactions—histamine, SRS-A, and ECF-A<sup>5</sup>. These are secreted after antigen challenge of basophils





**Fig. 1** The release of histamine and TAME esterase activity from human leukocytes. *a*, Dose-response relationships of antigen-induced release. The precision of the esterase assay is indicated by the standard deviation shown in the release curve caused by rye grass group I antigen (left-hand curve). The other two frames represent *b*, release by highly specific anti-IgE; *c*, kinetics of the release of the esterase (●) and histamine (○).

and mast cells sensitised with IgE antibody<sup>6-7</sup>. The release depends on calcium and temperature, requires energy and is controlled by hormone-receptor interactions which influence the intracellular level of cyclic nucleotides<sup>10-13</sup>. We have now demonstrated the immune release of an enzyme from human leukocytes that hydrolyses *p*-toluene sulphonyl-L-arginine methyl ester (TAME) and generates bradykinin from citrated human plasma. The release is initiated by the interaction of antigen or anti-IgE with cell-bound IgE<sup>6,14</sup> and seems to be similar in mechanism to the release of histamine and the other mediators of the allergic response.

Human leukocytes from donors allergic to ragweed or grass were separated from the other formed elements of the blood, washed free of serum and suspended in a serum-free Tris-buffered medium containing 0.03% human serum albumin, calcium and magnesium as previously described<sup>10</sup>. The immunological reaction was initiated by addition of antigen or anti-IgE to the cell preparations and the reaction proceeded for 30–45 min in the dose-response studies or for increasing periods in the kinetic studies. At the completion of the reaction, cells were centrifuged and the histamine released into the supernatant as well as that present in samples of untreated cells was determined spectrophotofluorometrically<sup>10</sup>. Arginine esterase activity of the supernate was determined radiochemically using <sup>3</sup>H-TAME, a method which was devised by Beaven *et al.*<sup>15</sup> to measure human urinary kallikrein and modified for determination of prekalikrein<sup>16</sup> and arginine esterase activity in supernatants<sup>17</sup>. The total cellular arginine esterase activity was determined using sonicated samples of untreated cells. Bradykinin was generated by treating citrated human plasma with the supernatant from cells challenged with antigen or anti-IgE and measured by a radioimmunoassay with a sensitivity of 4.0 ng as previously described<sup>18</sup>.

The dose-response relationships of the release of histamine and TAME esterase have been studied with leukocyte preparations from more than 20 allergic individuals challenged with either the purified protein antigens from ragweed (AgE)<sup>19</sup> or grass (Gp I)<sup>20</sup> or with highly specific anti-IgE<sup>21</sup>. Typical dose-response curves are shown in Fig. 1. The precision of the assay for histamine is 5–10% while that for the TAME esterase is about 3%, as shown in Fig. 1*a*. In general the pattern of the dose response curves for histamine and esterase release were similar, whether release was initiated by antigen (Fig. 1*a*) or anti-IgE (Fig. 1*b*). The absolute percentage of histamine or TAME esterase released by a leukocyte preparation may differ.

Rates of histamine and esterase release are compared in Fig. 1*c*. The time courses were essentially identical: this was confirmed in eight experiments. Further experiments demonstrated that the reaction did not proceed in the absence of calcium ions or at 4 °C. Moreover, the addition of EDTA or lowering the reaction temperature immediately stopped the release of esterase at any point in the reaction, as described for histamine release<sup>10</sup>.

The interaction of the TAME esterase with the kinin-generating system was studied. Using kaolin as a positive control to generate prekallikrein activator from Hageman factor it was observed that the supernatant from antigen-stimulated cells was inactive; no additional TAME esterase activity was noted after its interaction with plasma. This suggests that the leukocyte enzyme does not function as or cause the production of any prekallikrein activators. It does, however, generate kinin activity from citrated human plasma. In four experiments 30–350 ng of bradykinin was produced after incubation of the esterase with plasma for 20 min; buffer incubation with the same plasma yielded less than 4 ng of bradykinin (the limit of the assay). In a typical experiment the esterase or buffer was incubated with plasma in duplicate and the kinin generated was assayed in quadruplicate. The buffer control was below the limits of detection (< 4 ng) in each case whereas the esterase-treated plasma had  $33.4 \pm 3.2$  ng of bradykinin. Preliminary experiments have shown that the esterase can generate high levels of bradykinin from highly purified kininogen. It would appear, therefore, that the release of the TAME esterase is associated with kallikrein activity. Whether the two activities are subserved by the same enzyme, however, remains to be ascertained.

The parallelism in dose-response and kinetics of kallikrein and histamine release, as well as the release by anti-IgE, indicates that the kallikrein is derived from human basophils: previous work has demonstrated that basophils constitute the only type of white blood cell that fixes IgE and contains histamine<sup>22</sup>. Phagocytosis by polymorphonuclear leukocytes, induced by latex beads or immune complexes, releases some materials with protease activity but these are not kallikreins<sup>23</sup>. This, therefore, seems to be the first time that the generation of kallikrein from human leukocytes has been observed as a result of a primary immune reaction, and represents an important link between reactions of immediate hypersensitivity and the plasma kinin generating system. The demonstration of the IgE-mediated release of a basophil kallikrein allows us to begin to study the role of this system in the pathogenesis of acute inflammatory responses.

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- Colman, R. W., *New Engl. J. Med.*, **291**, 509 (1974).
- Brocklehurst, E. W., and Lahiri, S. C., *J. Physiol., Lond.*, **165**, 39P (1962).
- Jonasson, O., and Becker, E. L., *J. exp. Med.*, **123**, 509 (1966).
- Wintroub, B. U., Spragg, J., Stechschulte, D. J., and Austen, K. F., in *Mechanisms in Allergy* (edit. by Goodfriend, L., Sehon, H., and Orange, R. P.), 495 (Dekker, New York, 1973).
- Austen, K. F., in *Immunological diseases*, second ed. (edit. by Samter, M., *et al.*), 332 (Little, Brown and Co., Boston, 1971).
- Austen, K. F., and Brocklehurst, W. E., *J. exp. Med.*, **113**, 541 (1961).
- Kay, A. B., Stechschulte, D. J., and Austen, K. F., *J. exp. Med.*, **133**, 502 (1971).
- Lichtenstein, L. M., in *Biochemistry of the Acute Allergic Reactions* (edit. by Austen, K. F., and Becker, E. L.), 153 (Blackwell, Oxford, 1968).
- Grant, J. A., and Lichtenstein, L. M., *J. Immun.*, **112**, 897 (1974).
- Lichtenstein, L. M., and Osler, A. G., *J. exp. Med.*, **120**, 507 (1964).
- Orange, R. P., and Austen, K. F., in *The Biologic Role of the Immunoglobulin E System* (edit. by Dayton, D.) (US Government Printing Office, Washington DC, in the press).
- Lichtenstein, L. M., and Margolis, S., *Science*, **161**, 902 (1968).
- Lichtenstein, L. M., and DeBernardo, R., *J. Immun.*, **107**, 1131 (1971).
- Lichtenstein, L. M., *J. Immun.*, **107**, 1122 (1971).
- Beaven, V. H., Pierce, J. V., and Pisano, J. J., *Clin. chim. Acta*, **32**, 67 (1971).
- Imanari, T., Pisano, J. J., and Pierce, J. V., *Fedn Proc.*, **32**, 846 (1973).
- Webster, M. E., *et al.*, *Ciencia E Cultura*, **26**, 372 (1974).
- Talamo, R. C., Haber, E., and Austen, K. F., *J. Lab. clin. Med.*, **74**, 816 (1969).
- King, T. P., Norman, P. S., and Connell, J. T., *Biochemistry*, **3**, 458 (1964).
- Marsh, D. G., Milner, F. H., and Johnson, P., *Int. Archs Allergy*, **29**, 521 (1966).
- Ishizaka, T., Soto, C. S., and Ishizaka, K., *J. Immun.*, **111**, 500 (1973).
- Ishizaka, T., DeBernardo, R., Tomioka, H., Lichtenstein, L. M., and Ishizaka, K., *J. Immun.*, **108**, 1000 (1972).
- Movat, H. Z., Steinberg, S. G., Habal, F. M., and Ranadive, N. S., *Lab. Invest.*, **29**, 669 (1973).

# reviews

THIS book\* must fascinate everyone interested in the history of British zoology in the 19th century. It begins with Joseph Banks still in the seat of power, his soirées at 32 Soho Square attended by eminent scientists discussing the latest 'curiosities'. Over his shoulder one has a glimpse of Hans Sloane whose collections, gradually disintegrating, were housed in subterranean excavations, said to resemble the catacombs of Palermo, in Montagu House. The greatest collections remained in possession of the landed gentry and nobility; even those offered to the Government were largely refused because of lack of space in which to keep them. Appointed by patronage, the miserably paid Keepers of Natural History had neither status nor influence.

The gradual changes which led to the organisation of the greatest collections in the world occupied the whole of the Victorian era and were the work of two keepers, John Edward Gray and Albert Gunther, their lives and work here admirably surveyed by the grandson of the latter. The major events are the erection behind Montagu House of the British Museum with the transfer there of the Natural History collections in 1840 and 1841 and the far greater move, in distance and bulk of material, from Bloomsbury to South Kensington in 1882 and 1883.

Born in 1800, the son of a struggling botanist and pharmacist, John Gray proceeded by devious occupations into medicine, to emerge, by virtue of character and ability, as a leading naturalist. Friendship with W. E. Leach, then too briefly at Montagu House, introduced him to Banks and his circle. His first Museum employment, at a daily payment of 15 shillings, was during the keepership of J. G. Children whom he succeeded in 1840. Initially, with his fellow keepers he was subject only to the Principal Librarian with direct access to the Trustees, this changing in 1856 when Richard Owen was appointed superintendent of all the natural history departments.

The spirit of Linnaeus, tempered now by that of Cuvier, prevailed and the great task presented to Gray was



The new Coral Room

## Recollections of a century

the accumulation of specimens with "the diffusion of instruction and rational amusement". He travelled widely, visiting museums and meeting their directors and staff. Material began to pour in particularly from the colonies; collections of birds were purchased from John Gould, collections of shells from Hugh Cumming. Gradually, the collections came to rival in extent that of the still growing empire, although the Admiralty and Foreign Office were to disregard its claims until the conflict over the *Challenger* collections led to the final deposit of these in the Museum.

The first British Museum catalogues were contained in the suitably named *Naturalist's* (later *Zoologist's*) *Miscellany*, begun in 1790 by Shaw and illustrated by Nodder, then briefly continued by Leach. Gray's achievement was to produce the first adequate catalogues of major groups, starting with the reptiles. But how could a handful of underpaid, badly housed assistants contend with the flood of incoming material? Wallace's complaint to Darwin about the reaction to his claim to have brought 8,000 new species from the Amazon was met with commiseration for their overwhelming task and with a feeling that "too much systematic work and description blunt the faculties".

It was to produce more order out of chaos that the young Albert Gunther, initially a theology student at Tübingen, came to the Museum in 1857 to be led into "A suite of three half subterranean rooms, in which the spirit collection

was stored . . . The floor showed large damp patches. There was no ventilation. Non-inviting rheumatically quarters at any time. But what did I care?" What indeed?—he regarded this first year working on the great *Catalogue of Fishes* (1859–1870) as the happiest in his life. His later friendship with Alfred Newton at Cambridge bore fruit for the enduring sustenance of all zoologists by the establishment in 1856 of the *Zoological Record*.

Carrying the weight of the Department during Gray's final years of illness, Gunther succeeded him in 1875. His relations with Owen are interesting. Initially very much the protégé, differences developed alongside increased responsibilities, notably in connection with the new Museum at South Kensington for which Owen was fully responsible although the final plans were made without the knowledge of the keepers (or indeed of Owen himself). This is no place to discuss either the architecture or the internal planning of a building into which Gunther had to move specimens for the custody of which he was responsible although "their selection and arrangement was to be left to the Superintendent."

That figure was almost immediately replaced by the first appointed director, William Flower, who also assumed the keepership when Gunther retired in 1895, to be succeeded in this capacity by Sydney Harmer with Tate Regan as a junior member of staff. Here this book ends—not far short of when zoologists of my generation came into the picture.

C. M. Yonge

\* *A Century of Zoology at the British Museum—Through the Lives of Two Keepers*. By Albert E. Gunther. Pp. 533+37 photographs. (Dawsons: London, 1975.) £17.30.



## Quantum theory

*Quantum Theory of the Solid State.* By Joseph Callaway. Part A: Pp. x+1-370+10. Part B: Pp. xiii+371-824. (Academic: New York and London, July 1974.) \$29.50; £14.75.

THIS work is in two parts, the first containing an account of the formalism required to study solids and the second concerned with more specific problems. The book is at a level suitable for students with a good background in quantum mechanics and some knowledge of the experimental facts of the solid state.

Part A is divided into four chapters, the first on lattice dynamics being followed by magnetism at a phenomenological level. Chapters 3 and 4 can be viewed together as an introduction to the group theory, followed by the calculational methods needed to find the electron states (and from the symmetry standpoint the phonon states) in periodic crystals. Part B then treats impurities, the effect of external electric and magnetic fields on solids and transport phenomena. In the final chapter there is an introduction to many-body theory.

Professor Callaway, of course, has made important contributions over a wide area of solid state physics and the book reflects his deep understanding of his subject. I wonder, however, a little about his ordering of the material. For, although many-body theory is treated in depth only in the last chapter, on p. 22 we have the creation and annihilation operators, and on p. 41, also in the first chapter, the van Hove correlation function appears in its full quantum mechanical form. Nevertheless, even for the reader who does not struggle too hard with the more advanced detail in Chapter 1, there is a good deal of basic solid state physics concerned with phonons which can be learned from it. The references, which are at the end of each chapter, number about 60 on phonons. Three refer to work later than 1971 but the rest are from 1969 and earlier.

Chapter 2 begins with the Heisenberg Hamiltonian along with some discussion of how the exchange parameters can be calculated from first principles. Molecular field theory is then discussed at some length, followed by an account of antiferromagnetism. The spin wave problem is then tackled using the Heisenberg Hamiltonian, with the dependence of spin waves on an external magnetic field given some attention. The extensive and helpful (though pretty advanced) discussion of spin waves is followed by a discussion of neutron scattering from magnetic crystals. The Ising model is then given

a good deal of prominence, 15 pages or so being devoted to it. Phase transitions in ferromagnets are then treated, at about the same length. The discussion is scholarly, but in my opinion rather more advanced than is consistent with the author's remarks in the preface about the level of students it is written for.

In the chapter on symmetry, one of the nice features is the attention given to crystal field effects. The reader will probably find Chapter 3 on symmetry and Chapter 4 on band theory a good deal easier than the first two chapters. The chapter on energy bands has all the clarity one would expect from such an expert in the field; however, quite a bit of the discussion is available already in good sources. The discussion of the tight-binding method, which Professor Callaway has used to very good effect on the transition metals, is very helpful here. Finally, the central question of the way the crystal potential is to be constructed is discussed from the standpoint of density functional theory; Dirac-Slater exchange theory and gradient corrections to it are summarised.

Part B begins with the impurity problem, Wannier and also *t* matrix methods being dealt with in some detail. A nice account of the optical

theorem is to be found here. Effective mass theory, local moments, the coherent potential approximation and the Kondo effect are other topics treated. A chapter of about 100 pages then follows on crystals in external fields, including optical properties, and magnetic field phenomena, such as low field diamagnetism and the de Haas-van Alphen effect. Transport theory is discussed at length and the last chapter represents a useful introduction to many-body theory. Itinerant electron magnetism is treated in a very clear manner in this chapter, along with Landau Fermi liquid theory. Green's functions and diagrammatic analysis are also developed here.

The only general criticism I would want to level concerns the ordering of the material. The first two chapters might have been better placed after the consideration of symmetry and band theory. More discussion of many-body theory would have been helpful earlier in the book.

Without doubt, this is a valuable addition to the literature which I found very stimulating to read. The problems at the end of each chapter are helpful in assessing one's grasp of the material as one goes along, though some are pretty difficult.

N. H. March

## Sound through the sea floor

*Physics of Sound in Marine Sediments.* Edited by Loyd Hampton. Pp. xii+567. (Plenum: London and New York, 1974.) \$39.00.

THIS publication is a collection of 20 papers presented as the proceedings of one of four symposia organised by the Office of Naval Research to review current research activities and to discuss possible future trends in the subject of sound propagation in marine sediments. Edited by Loyd Hampton, the papers describe the research efforts of many of the world's leading authorities on a subject which is of increasing importance as the commercial exploitation of the natural resources that lie on, or beneath, the sea floor continues to expand into less accessible areas.

This collection of papers is presented quite informally and is therefore comprehensible to those not too familiar with all of the aspects of a relatively diverse field. The volume highlights the difficulties involved in obtaining accurate geophysical, acoustic and mechanical data from sediments in their natural environment. Such data have perhaps, not been given sufficient priority in the past, but the advent of large structures that are to be placed on the sea floor may change that situa-

tion. Current static loading tests may soon prove inadequate in solving settlement problems in the high energy environments that exist at sea. A possible alternative may be to consider the interrelationships between the required parameters and such physical properties as sound propagation, electrical resistivity and radioactivity. Acoustic properties will probably prove to be the most useful in this respect because of the fundamental relationships involving mechanical and physical properties of materials. With knowledge of these relationships, acoustic measurements could combine with the normal methods of site investigation which use standard acoustic techniques to provide more accurate interpretation as well as dynamic loading information. Continuous monitoring of the acoustic properties of sediments may also allow the measurement of the long term changes in sediment fabric and of the effects of severe weather conditions on sediment structure.

The presentation of the papers is of a general high standard, but as many authors are involved it is often necessary to compare data; this task would have been simplified if a standard set of units had been specified.

Peter G. Simpkin

## Wrong word too early

*Invertebrate Endocrinology and Hormonal Heterophyly*. Edited by Walter J. Burdette. Pp. xviii+438. (Springer: Berlin and New York, 1974.) \$21.50.

HETEROPHYLLY, as defined by the Oxford Dictionary, is a botanical term referring to plants having leaves of more than one shape. How then does this relate to invertebrate hormones? In fact, it does not. The editor has given a new meaning to the word, and uses it to describe the effects of hormones from one phylum on individuals of another phylum. From the title one expects to read about hormones from various invertebrate groups. But, in truth, the book considers only insect hormones, and of these, only ecdysone, juvenile hormone, and brain hormone.

The book contains contributions made to a meeting held at Houston early in 1971. Unfortunately, there has been a long delay between the submission of the manuscripts and the appearance of the book in late 1974, and, consequently, it is rare to find a reference later than 1970; in fast moving areas of research, such as juvenile hormone and ecdysone biosynthesis and degradation, many papers are already out of date.

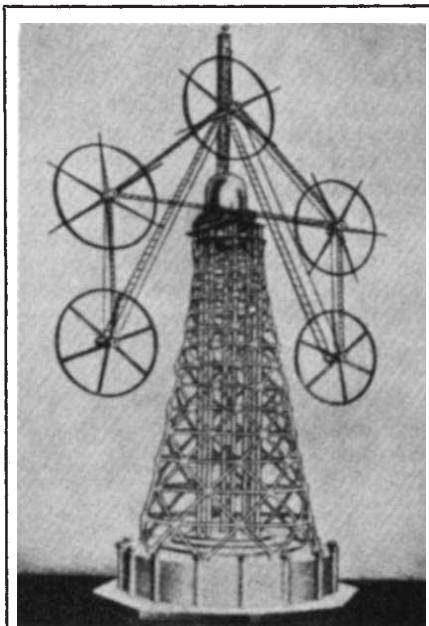
The book is divided into four parts. Part I, "Invertebrate endocrinology", deals with the control of metamorphosis, hormone assays, and hormone biosynthesis and degradation. The section starts promisingly with remarks by Wigglesworth on the use of hormones to probe basic problems in the regulation of growth and differentiation. The following papers include articles that cater to a general audience as well as those with very technical information aimed at the specialist. The series of papers on assay techniques, an example of the latter, are varied in their usefulness. The paper by Bjerke and Roller on juvenile hormone assays provides a broad, critical comparison of the strengths and weaknesses of the common bioassay systems that were in use at the time. By contrast, the treatments of the brain hormone and ecdysone assays are much more limited in their scope and, accordingly, are of less value.

The second part considers primarily the isolation and distribution of various ecdysones. Since the original report in 1966 by Nakanishi and his coworkers of the presence of ecdysone-like chemicals in plants, the isolation and identification of these substances has, with a few notable exceptions, been carried out by workers in Japan. The articles in this section serve the valuable function of bringing together the data from the Japanese literature on the structure of the known phytoecd-

sones (37 at the time the paper was written) as well as extensive listings of their plant sources.

Part III comprises six papers on the effects of vertebrate hormones on vertebrate tissues. Except for presumed parallels between the mode of action of these hormones on vertebrates, and that of insect hormones on insects, these papers bear little relationship to the rest of the book and seem to be quite out of place.

Hormonal heterophyly is the subject of the last part. From the various articles, it quickly becomes apparent that the effects of insect hormones on



Wind turbine designed in 1933. It was to have stood 300 metres high and would, according to its designer, have generated 50,000 kW. From *Power From the Wind*, by Palmer Cosslett Putnam. Pp. xii+224. (Van Nostrand Reinhold: New York and London, October 1974.)

vertebrate cells are neither striking nor profound. The effects of ecdysone on the growth of mammalian tumours and on human leucocyte chromosomes are at best slight and are certainly of questionable statistical significance. The insect hormones do cause metabolic changes in mouse liver, but this can scarcely be surprising in a tissue adapted for detoxification of foreign substances. Indeed, the fact that insect hormones have little effect on vertebrate tissues, as documented by the papers dealing with pharmacological screens of these hormones, is a point in their favour. Especially as one day they may be released into the environment as insecticides.

The editor of this volume states that it is the first concerted effort to collect and analyse responses of vertebrate, as well as invertebrate, tissues to insect hormones. In my opinion, it is a premature attempt. **James W. Truman**

## Old bones

*Bones for the Archaeologist*. By I. W. Cornwall. Revised edition. Pp. 259. (Dent: London, February 1975.) £4.50.

THIS is a revised edition of a volume first published in 1956. The section on conservation has some useful additions, the bibliography and the references have been improved and brought up to date and a very necessary index has been added. The bulk of the text and the illustrations remain the same.

When this book was first published the intention was to help the archaeologist make preliminary identifications of bones from archaeological sites at a time when much data had accumulated in museums awaiting the interest of some specialists from the natural sciences. It demonstrated successfully that most of this work could be done by the archaeologist himself if he was prepared to apply himself to learning the basic necessities of bone analysis. Many archaeologists, who were not entirely artefactually orientated, found it both necessary and relatively easy to add to their competence. There can be little doubt that some improvement in excavation techniques was one of the consequences of the publication of their book, as well as a reduction in the backlog of material awaiting analysis and identification.

The volume contains chapters on zoological classification and nomenclature, bone identification, field work, laboratory work and conservation, the estimation of age, sex and stature, and study and interpretation. For the tiro it even suggests logical methods of procedure. It is easily the best introductory work available at the present time and will guide many newcomers to archaeology to a useful and essentially practical expertise.

On the other hand, the weakest section is that concerned with study and interpretation. There is no clear indication of what purposes underlie the collection of bone specimens nor, in consequence, how bones should be collected on the site to give an adequate sample. It still remains that, in contrast to pollen analysis, data is atrociously collected during excavation by archaeologists with vastly different interests, techniques, knowledge and relevant expertise; there is still no discipline about archaeological collection. In consequence, samples obtained are commonly of minimal value and amenable to valid interpretation only at the coarsest levels. This fact could have been pointed out to advantage at a time when numerical analyses are increasingly fashionable. Excellent teaching as, for the most part, it is, Dr Cornwall's book stays with the vision of some 20 years ago. **E. S. Higgs**



# obituary

**John E. Vance**, a chemist who worked on processing of ores and the preparation of pure uranium compounds for the Manhattan Project, has died in New York at the age of 69.

Dr Vance was emeritus professor of chemistry at New York University and former head of the department. He joined the faculty in 1948 and retired in 1971. He had previously been associate professor at Yale, where he directed work on quantitative analysis, and crystal growth. In the 1940s, he was appointed by the State Department as senior staff member to Frederick Osborn, representative to the United Nations Atomic Energy Commission. Dr Vance's special concern was drawing up proposals for international control of atomic energy. At various times, he had served the army research office, particularly during the Manhattan Project which developed the atomic bomb in 1942.

**Cyril Leslie Oakley, CBE, FRS**, the distinguished bacteriologist, died on March 27 at the age of 77.

Professor Oakley graduated from University College, London, in Zoology in 1930 and held the position of experimental pathologist at the Wellcome Research Laboratories from 1934, becoming head of the Immunology and Experimental Pathology Department in 1947. From 1953, he was Brotherton Professor of Bacteriology at the University of Leeds, becoming Professor Emeritus in 1972. His own interests were in the fields of antibody production and bacterial toxins, and looked after those of others as President of the Association of Scientific Workers from 1956–59. Professor Oakley will, however, be primarily remembered as the editor of a number of scientific journals, including *Pathology and Bacteriology* (1956–68), *Medical Microbiology* (1968–71) and *Pathology* (1969–72).

**Louis W. McKeehan**, professor of physics at Yale from 1927–55, has died in Jamestown, Rhode Island at the age of 87.

After graduating from the University of Minnesota in 1908, Professor McKeehan taught there as an associate professor until the outbreak of war. At Yale, he directed the Sloane Laboratory of Physics and after becoming emeritus professor, he was research associate at the Laboratory of Marine Physics in New Haven and Jamestown. He had a lifelong association with the navy and had been assistant naval attaché in London during World War II and chairman of the scientific advisory board of Operation Crossroads, for the strategic bombing of Japan. His research included discharge in gases, ferromagnetism, submarine mines, slow-motion spheres in gases, scattering of cathode and  $\beta$  rays, and radioactivity.

## announcements

### Awards

**George J. Todaro** has been awarded the **Parke, Davis Award** by the American Society for Experimental Pathology for investigation of viral and genetic factors in the cause of cancer, in particular the viral oncogene hypothesis.

**H. E. Ganther** has been awarded the **Mead Johnson Award** by the American Institute of Nutrition for his work on the essential nutrient selenium.

### Appointment

**J. G. Williams** has been appointed professor of polymer engineering at Imperial College, London.

### International meetings

May 28–30. **Frequency control**, Atlantic City (Dr J. R. Vig, US Army Electronics Command, Fort Monmouth, New Jersey 07703).

June 2–5, **Information transfer in eukaryotic cells**, Montreal (Ms D. Mathieu-Thériault, c/o Dr R. Sinclair, Biology Department, McGill University, Box 6070, Montreal H3C 3G1, Canada).

June 7–18, **Solar terrestrial physics**, Colorado (American Geophysical Union, 1909 K Street, NW, Washington, DC 20006).

### Miscellaneous

**Cancer awards.** The Cancer Research Institute Inc., is establishing Annual Awards in Cancer Immunology. The Institute will honour work judged to have been most critical in founding Cancer Immunology as we know it today, whether in reference to basic experimental research or to observations of more immediate clinical significance. Nominations to Mrs Helen C. Nauts, Executive Director, Cancer Research Institute Inc., 1225 Park Avenue, New York, New York 10028 (closing date July 15, 1975).

**Cherwell-Simon Lecture.** The lecture for 1974–75 will be delivered by E. W. Montroll, Director of the Institute for Fundamental Studies and Albert Einstein Professor of Physics in the University of Rochester, New York, on May 23, at 4.30 pm in the lecture theatre of the university museum. The subject will be 'Some quantitative aspects of social phenomena'.

### Reports and publications Great Britain

Parliamentary and Scientific Committee. Annual Report for 1974. Pp.16. (London: Parliamentary and Scientific Committee, 1975.) (112)

Ministry of Agriculture, Fisheries and Food. Novel Protein Intelligence Unit. Bulletin No. 3: Leaf Protein—Current Developments in the United Kingdom. Pp.28. (London: Ministry of Agriculture, Fisheries and Food, 1975.) (112)

Department of Industry. Report on Research and Development 1974. Pp.122. (London: HMSO, 1975.) £1.10 net. (122)

The Association of Commonwealth Universities. Annual Report of the Council together with Accounts of the Association for the year 1 August 1973 to 31 July 1974. Pp.52. (London: The Association of Commonwealth Universities, 1975.) (122)

National Institute for Medical Research. Report 1973/1974. Pp.iii+123. (London: Medical Research Council, 1974.)

Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 277, No. 1274: Analysis of the Flexural Vibrations of Variable Density Spheroids Immersed in an Ideal Fluid, with Application to Ship Structural Dynamics. By R. E. Taylor. Pp.623–648. (London: The Royal Society, 1975.) £1. (132)

### Other countries

Académie Royale de Belgique. Mémoires de la Classe des Sciences. T.XLI, Fasc.6: Contribution à l'Etude de l'Atmosphère Terrestre Supérieure à Partir de l'Analyse Orbitale des Satellites. Par J. Vercheval. Pp.183. (Bruxelles: Académie Royale de Belgique, 1974.) (252)

Cadmium and the Environment: Toxicity, Economy, Control. Pp.88. (Paris: Environment Directorate, Organization for Economic Co-operation and Development, 1975.) (262)

La Théorie des Pulsations Internes de la Terre: Compléments. Par Georges Dubouvier. Pp.47. The Theory of Internal Pulsations of the Earth: Further Evidence. By Georges Dubouvier. Translated from the French by Louis Janssens. Pp.49. (Meudon: Collège de France, Laboratoire de Géologie, 1974.) (000)

Alberta Research Council. Annual Report 1974. Pp.67. (Edmonton: Alberta Research Council, 1975.) (282)